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The cover, taken from the first manuscript published in this issue, is an ascomycete (Xylariales) mycelia with fruiting bodies isolated from buried plastic sheet (Type II).

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Isolation of Decomposer Fungi With Plastic Degrading Ability[1]

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ABSTRACT

A study was conducted to isolate from natural environment decomposer fungi that has the capability to degrade plastic sheets. The isolates will be used as component of a new mixed fungal inocula for rapid composting of market wastes. Rate of decomposition of four different types of plastic bags in the natural environment was also determined by measuring their loss in weight with time. Mean half-life of plastic bags was computed.

Ordinary polyethylene sando plastic bags commonly used as containers of consumer goods in dry goods stores and wet markets were buried 2 cm deep on the forest floor. Each sample was cut on two sides and converted into flat sheet before burying. At regular monthly intervals the plastic sheets were retrieved, cleaned of adhering soil particles and artifacts and plated in coconut water agar. Fungi growing on the plates were isolated and identified. Plastic degrading capability of the isolates were tested by growing them in mineral medium where plastic strips were used as main source of carbon. Assessment of the plastic degradation was measured in terms of loss in weight of the plastic strips and by the amount of mycelia produced by the fungal isolates.

There were four species of microfungi found growing on the plastic sheets. They were two species of Penicillium, one species of Aspergillus and sterile mycelia belonging to Ascomycete Xylariales group. Only Ascomycete mycelia developed good growth in all the treatments with only plastic strips as source of carbon. This isolate also caused decreases in weights of the plastic strips in these treatments. This isolate is now a component of the fungal inocula being pilot tested for composting market wastes.

This study is funded by the IBS Basic Research.

This study also showed that even the thinnest and most transparent form of plastic bag has a mean half-life of 14.5 years with range of 6 - 29 years. This means that the simplest plastic bag will take about 100 years to be completely decomposed. This slow rate of decomposition may be due to very few decomposer organisms that can attack the carbon atoms present in the plastic sheet. There is a need to rationalize the use of plastic bags in our daily life.

INTRODUCTION

Plastics have been part of our modern day living. With the growth of the Petro Chemical Industries after World War II, various forms of plastic products were manufactured from naphtha fractions of petroleum or from natural gas. These plastic products have come to replace a number of natural products such as wood, glass or even metal as construction materials and paper bags as wrappers of consumer goods.

Plastic products have many industrial and commercial applications. They are cheap, light, water impermeable, non toxic, chemically stable, biologically inert and durable. These properties make these products popular to our modern society.

This popularity of plastic products pose considerable environmental problems. Although there are recycling plants for discarded plastic products in the country, there is still considerable work to be done for organized collection in municipalities and cities so that discarded plastic products will reach these recycling plants. In addition, there are some plastic products that can not be recycled and pose disposal problems like the plastic bags that are normally used as wrappers and packaging materials. In some countries like Japan, Sweden and Switzerland, these non-recyclable plastic products are burned in incinerators to generate steam, hot water or electricity (Cain, 1992). In our country discarded plastic products have become nuisances and are scattered all over the places making them unsightly. They mostly clog canals, drainage systems and rivers impeding free flow of water.

The most commonly used plastic bags as wrappers and packaging materials in our country are synthetic, thermoplastic, polyethylene polymers which melt when heated. These polymers are long chain hydrocarbons of the alkane type. Polyethylene (PE) has a chain length orders of magnitude larger than the largest naturally occurring alkane consisting of chain length between 70 and 71000 C atoms (Cain, 1992). Low density PE has a limited amount of chain branching and general of shorter chain length.

This study was conducted to isolate decomposer fungi observed to be growing in plastic bags commonly used as wrappers in wet markets. The main idea is to incorporate these isolates as component of mixed fungal inocula for rapid composting of market wastes, similar to the compost fungus activator (CFA) developed by the senior author for composting agricultural wastes (Cuevas, et.

al. 1988). The study was also conducted to determine the rate of decomposition of these plastic bags in natural environment. The overall objective of the study is develop strategies for solid waste management.

METHODOLOGY

A. Isolation of fungi growing in plastic bags buried in forest floor

A set of sando plastic bags commonly used as wrappers in wet markets were laid on separate locations on the forest floor of UPLB Hortorium. These bags are polyethylene plastics (PE) that are cheap and easily available. The bags were opened on two sides such that one layer plastic sheets were in contact with the soil. The plastic sheets were collected every two months for six months. After harvest the sheets were cleaned gently of adhering debris, washed thoroughly with sterile distilled water, cut into pieces and plated (by dilution pour plating) in sterile coconut water agar (CWA)plates. Fungi found growing on the agar plates were isolated and identified.

B. Test for the plastic biodegrading ability of the isolates

The fungal isolates were tested for their ability to degrade plastic materials under laboratory conditions. Biodegrading ability was measured in terms of a) mycelial dry weights they produced in culture medium; b) the loss in weight of the plastic strips after the fungal growth has been removed from the surface of the materials.

Each isolate was grown in Erlenmeyer flask with 50 ml mineral medium (See Appendix A for composition of medium) where carbon source has been replaced with 50 mg plastic strips (1 cm X 1 cm). These plastic strips were cut from the same type of bags that were used in isolating the fungi. Their weights were taken before use in the experiment.

Appendix A.

Mineral medium used in test for plastic degrading ability of isolates

Carbon source	0.1 g NaCl
5.0 g ammonium tartrate	0.01 g $FeCl_3$
1.0 g Difco malt extract	5.0 ml vitamin solution (1% thiamin solution)
0.5 g $MgSO_4 \cdot 7H_2O$	1000 ml distilled water
0.01 g $CaCl_2 \cdot 2H_2O$	

(Adopted from Sundman, V. and L. Nase, 1971)

After one month incubation at room temperature the fungal mycelium was gently scraped off from the plastic strips, placed in pre-weighed filter paper, dried and weighed. The plastic strips were washed with sterile distilled water, air dried and re-weighed. One species of Trichoderma which has been proven to have good cellulase activity was included in this test as control. Four treatments with 4 replicates per treatment were made:

T1 - 50 mg plastic strips + 0.5% glucose as C source
T2 - 50 mg plastic as main source of carbon
T3 - 2.0% glucose as C source - Control treatment
T4 - 50 mg plastic + 0.05% Ca $(NO_3)_2$ - (to replace the malt extract as N source)

C. Measurement of rate of decomposition of plastic bags

Four types of bags commonly used in markets and sari-sari stores as containers of consumer goods were selected. (One of the four types of bag used in this part of the study was used in parts A and B). Twenty four unused samples of each type of plastic bag were utilized in the study. Each of these bags was cut at the sides to form single sheet, air dried and initial weights taken. These plastic sheets were buried 2 cm deep into soil and forest litter at the forested area of UPLB Hortorium. At two months interval, four replicates of each plastic type were collected, cleaned gently of adhering soil particles and other debris, air dried and weighed. Physical appearance and characteristics of the plastic sheets were noted. The samplings were done for one year. Decomposition was measured in terms of loss in dry weights of the plastic sheets. Rate of decay was calculated based on the equation of Weigart and Evans (1964) as cited by Cuevas and Sajise, 1978:

$r = \frac{\ln W_o/W_t}{t}$ where W_o = mean initial weight of plastic bags
W_t = mean weight of bags at each sampling date
t = time in months

RESULTS AND DISCUSSION

A. Fungi isolated from plastic materials

There were four different types of microfungi isolated from the buried sando plastic materials. These fungi were identified as Penicillium lilanicum (= Paecilomyces lilanicum), Penicillium sp. (belonging to Pen. notatum series), Aspergillus sp. and sterile dark mycelia. The sterile dark mycelia did not produce any sexual or asexual fruiting body. However close examination of the isolate the mycelia clearly resemble that of the Ascomycetes Hypoxylon and Xylaria. This type of sterile mycelia were found by Cuevas and Uyenco, 1977 to be common mycoflora of decomposing leaf litters. Penicillium sp. was also found growing in plant litters in later studies.

Figures 1, 2 and 3 show the morphology of these isolates.

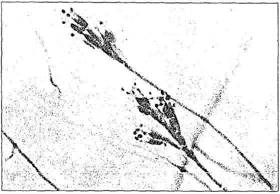

Figure 1. Asexual reproductive structures of Penicillium lilanicum (=Paecilomyces lilanicum) isolated from buried plastic sheet (Type II).

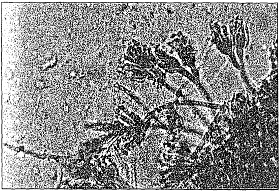

Figure 2. Asexual reproductive structures of Penicillium sp. isolated from buried plastic sheet (Type II).

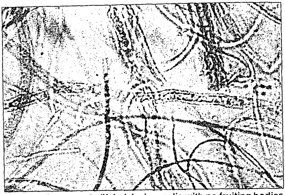

Figure 3. Ascomycete (Xylariales) mycelia with no fruiting bodies isolated from buried plastic sheet (Type II).

There are reports in literature that some species of fungi are able to grow on biodegradable polymers. Tokiwa and Suzuki, 1974 as cited by Schonborn, 1986 has shown that a species of Penicillium was able to degrade polyethylene glycol adipate. The same author has reported that a range of fungi has been isolated from polyurethanes. These fungi were able to cause changes in tensile strength and modulus of elasticity of the materials. When buried in the soil, these polyurethane materials have thin film crack, and are brittle and suspected that the stress cracks were results of the initial weakening of the structure by the fungi. Aspergillus versicolor and a Fusarium species have been reported as important agents of biodeterioration of p.v.c. plasticizers.

There are also reports that 28 species of fungi and 193 bacterial species were isolated from the surface of starch-polyethylene films (modified polyethylene materials) exposed to a leaf compost row (Gilmore, et. al., 1992 as cited by Johnson, et. al. 1993). White rot basidiomycetes are also common organisms used in testing the biodegradation of lignopolystyrene graft copolymers (Milstein, et. al., 1992).

However there were no reports on fungi or bacteria that can degrade or attack polyethylene materials such as the plastic bags.

B. Test for the plastic biodegrading ability of the isolates

Table 1 presents the results of the test on the ability of the isolates to grow and utilize plastic materials as carbon source. In this test T. harzianum IBS strain was included to determine if it has plastic degrading ability since there is a plan to include this organism as one of the component decomposer fungi to be used as activator for composting market solid wastes. Furthermore, Trichoderma species are cellulose degraders. Cellulose nitrate had been mentioned in literature as one type of component of plastic materials and therefore could be attacked by this species.

Table 1. Mycelial dry weights* and corresponding loss in weight of plastic strips* in various media inoculated with harzianum IBS strain, *penicillium* sp., *paecilomyces lilanicum* and ascomycete (Xylariales) mycelia.

Organism	Treatment 1		Treatment 2		Treatment 3	Treatment 4	
	mycelial dry wt. (mg)	loss in wt. of plastic (mg)	mycelial dry wt. (mg)	loss in wt. of plastic (mg)	mycelial dry wt. (mg)	mycelial dry wt. (mg)	loss in wt. of plastic (mg)
T. harzianum IBS strain	25.9	0.0	28.1	0.0	75.4	30.2	0.0
Penicillium sp.	57.5	0.0	23.9	2.7	89.5	39.2	0.0
Paecilomyces lilanicum	65.3	2.2	34.8	2.2	146.7	18.7	0.0
Ascomycete (Xylariales) mycelia	68.2	9.0	37.9	4.0	191.3	43.8	5.2

Treatment 1 - 50 mg plastic + 0.5% glucose as source of carbon
Treatment 2 - 50 mg plastic as main source of carbon
Treatment 3 - 2.0% glucose as source of carbon (control)
Treatment 4 - 50 mg plastic + 0.5% calcium nitrate

*mean of 4 replicates

As shown in the results this fungus is non plastic degrader. It did not grow in the different treatments where plastic strips were used as carbon source. Aspergillus sp. that was isolated from the plastic sheets was not included in this test since it has very low cellulase activity. As mentioned already, the study was conducted to be able to formulate a combination of decomposer fungi that will be used for rapid composting of market wastes which are mainly cellulosic.

Results of this test showed that three fungal isolates can degrade to a small extent the plastic strips. These organisms were able to assimilate carbon from plastic as shown by the loss in weights of the plastic strips and by the development of fungal mycelia. (see Figs. 4 and 5).

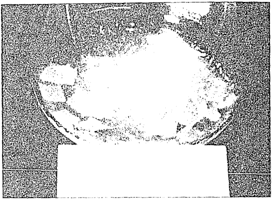

Figure 4. Growth of *Paecilomyces lilanicum* on medium with 0.5% glucose and 50 mg plastic strips as sources of carbon.

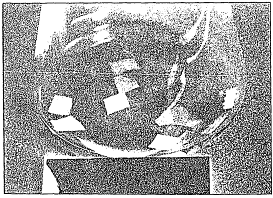

Figure 5. Growth of Ascomycete (Xylariales) on medium with 0.5% glucose and 50 mg plastic strips as sources of carbon.

Ascomycete mycelia after one month incubation caused about 9.0 mg loss in weight of plastic strips in the presence of small amount of glucose (Table 1- T1). Among the fungi isolated and tested this fungus caused the highest weight loss in all treatments with plastic strips. This fungus was the only isolate able to grow in T4, the medium where all sources of carbon were removed except the plastic strips. It caused loss in weight of

plastic strips of about 5.2 mg in this treatment. While the two penicillia did not cause any loss in weight of the plastic strip in T4, this Xylariales ascomycete had well developed mycelium and caused greater decrease in weights in T4 than in T2 where malt extract and plastic strips were present as C sources. These are evidences that showed this fungus was able to utilize the carbon from plastic and convert the element into protoplasm. It grew better in the presence of small amount of glucose and malt extract (T1) than without glucose (T2). The glucose and malt extract may been used as carbon sources for initial growth. However, the significantly higher mycelia produced by the organisms in the medium with glucose as source of carbon showed that plastic materials are not the prepared substrates.

It has been observed also in the lab as shown in figures 4 and 5 that the fungi grew more along the edges of the cut strips. It would seem that the fungi can attack the exposed surfaces along the edges. Thus in nature, the slow rate of decomposition can be still be partly hastened by mechanical breakdown such as shredding the plastic materials into smaller strips.

Schonborn, 1986 has mentioned that the presence of more easily assimilable carbon sources such as glucose or malt extract seemed to be necessary to initiate esterase activity to degrade the plasticizers in p. v. c. These plasticizers are mostly low molecular weights compounds and commonly organic esters. The plasticizers are the most susceptible to attack especially under tropical conditions of high temperature and high moisture. Brown, 1945 as cited by Eggins and Mills, 1971 stated that almost half of the known 144 plasticizers could served as fungal nutrients. These two authors have also demonstrated that in simulated biological refuse system, 9 out of 12 plasticizers were able to enhance the respiration of thermophilic soil microorganisms. Thus they have concluded that composting of municipal solid wastes is commendable as an alternative to incineration.

In this study, we were not able to further test which component molecule of the plastic strips were utilized by Ascomycete mycelia for its growth. Lack of funds and instrument like the Scanning electron microscope (SEM) prevented further work. This unfinished part of the study can be done in the near future.

However the main purpose of the study to isolate decomposer fungus that can be used as component of fungus inocula for biodegradation of market wastes was accomplished. Ascomycete mycelia is now a component of fungal inocula being pilot tested in a research project on market waste degradation supported by the Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD).

C. Rate of decomposition of plastic bags in natural environment

Table 2 presents the instantaneous rate of decay and half life of each type of plastic bag studied. As shown in the table, the rate of decomposition was very slow measured in terms of loss in weight as mg/mg/month. The rate was highly variable. The initial weights of the plastic bags also differed greatly even within the same plastic type depending upon their size and thickness. Among the plastic types studied the highest instantaneous rate of decays was registered in type I plastic bag, which was the thinnest and most transparent. It occurred during the first two months of incubation with an instantaneous rate of decomposition of 0. 015 mg/mg/month. All other types of plastic bags had a much slower rate

Table 2. Instantaneous rate of decomposition and half life of common plastic materials.

Plastic Type 1

Incubation Period (mos) (t)	Initial Weight (mg) (Wo)	Weight After Incubation (mg) (Wt)	Cummulative Loss in Weight (mg)	Instantaneous Rate of Decomposition (r)	Half Life (yrs)
2	154.25	150.55	3.70	0.015	3.85
4	170.00	165.20	4.80	0.008	7.22
6	151.75	144.50	7.25	0.008	7.22
8	168.50	162.40	6.10	0.005	11.55
10	166.50	163.30	3.20	0.001	57.75
12	154.20	146.80	7.40	0.004	14.44
Mean	160.87	155.46	5.41	0.007	17.00

Plastic Type 2

Incubation Period (mos) (t)	Initial Weight (mg)	Weight After Incubation (mg)	Cummulative Loss in Weight (mg)	Instantaneous Rate of Decomposition (r)	Half Life (yrs)
2	286.75	285.90	0.85	negligible	cannot be determined
4	312.00	308.00	4.00	0.003	19.25
6	309.75	294.00	15.75	0.010	5.78
8	288.00	282.60	5.40	0.003	19.25
10	290.00	283.80	6.20	0.002	28.88
12	303.80	290.00	13.80	0.004	14.44
Mean	298.38	290.72	7.67	0.004	14.60

Plastic Type 3

Incubation Period (mos) (t)	Initial Weight (mg)	Weight After Incubation (mg)	Cummulative Loss in Weight (mg)	Instantaneous Rate of Decomposition (r)	Half Life (yrs)
2	737.25	735.80	1.45	negligible	cannot be determined
4	752.50	748.80	3.70	negligible	cannot be determined
6	845.75	818.50	27.25	0.005	11.55
8	836.50	830.80	5.70	0.001	57.75
10	827.20	818.10	9.10	0.001	57.75
12	660.80	637.20	23.60	0.003	19.25
Mean	776.67	764.87	11.80	0.002	24.38

Plastic Type 4

Incubation Period (mos) (t)	Initial Weight (mg)	Weight After Incubation (mg)	Cummulative Loss in Weight (mg)	Instantaneous Rate of Decomposition (r)	Half Life (yrs)
2	860.50	857.50	3.00	0.005	11.55
4	870.00	864.40	5.60	0.003	19.25
6	862.75	839.00	23.75	0.005	11.55
8	855.30	851.60	3.70	negligible	cannot be determined
10	871.50	866.90	4.60	0.001	57.75
12	859.50	832.00	27.50	0.003	19.25
Mean	863.26	851.90	11.36	0.003	19.89

$$\text{Rate of Decomposition (r)} = \frac{\text{InW o - InW t}}{\text{t (mos)}} = \text{m g/mo}$$

where, Wo = mean initial weight of plastic bags
Wt = mean weight of bags at each sampling date
t = time in months

$$\text{Half Life } (t_{1/2}) = \frac{\ln 2}{r} \qquad \text{Half life of leaf litter (year)} = \frac{\ln 2}{r} = 0.4222$$

where, r = 0.0342 kg/ha/wk (Cuevas and Sajise, 1978)
= 1.6416 kg/ha/yr

of decomposition with the thicker bags having much slower rate of decay. Type II and Type III bags did not lose weight in the first months. The description of the different plastic bags used are given in Appendix B.

Appendix B.

Types of plastic bags used in measuring rate of decomposition of plastic sheets

Type I light, thin and transparent commonly used as wrappers of items bought in wet markets - (polyethylene bag]);

Type II - blue "sando" bag commonly used as replacement for the "bayong" of the olden days to carry various items bought in the market - (polyethylene bag);

Type III - yellow plastic bag thicker than Type I and Type II and normally used in supermakets - (polyethylene bag); and

Type IV - polypropylene bags

Figure 6 shows how the plastic sheet (Type II) looked after 4 months on the forest floor. The appearance of the plastic sheet showed that there has been decomposition of the test plastic materials. This conclusion is based on the definition of Hueck, 1965 as cited by Eggins, et. al. 1971, of biodeterioration as "any undesirable change in the properties of a material of economic importance caused by the activities of organisms". The buried plastic sheets had holes in them most probably caused by mechanical activities of microfauna. Eggins, et. al. 1971 mentioned that mechanical damage on plastics is caused by gnawing activities of termites, insects and rodents and larvae of some moth species.

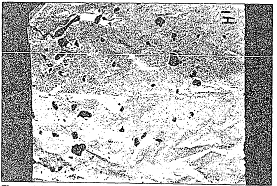

Figure 6. Plastic sheet (Type II) buried for 4 months in soil and litter.

As discussed in parts A and B of this article, there were also microfungi found growing on the plastic sheets. At least one fungus was found to be capable of using the plastic strips as source of carbon for their growth.

There are also many reports of species of bacteria like Pseudomonas acuruginosa ATCC 13388, causing losses in plastic strips buried in soil (Cain, 1992). Actinomyces belonging to *Fam. Streptomycetaceae* were reported by Klausmeier, et. al., to cause weight loss of plasticized polyvinyl chloride samples. As mentioned in the earlier discussion, these microbial attacks are in the plasticizer additives of p. v. c.

The mean rate of decomposition for all the plastic types ranged from 0.002 mg/mg/mo - 0.007 mg/mg/mo. These rates were tremendously slower as compared to the rate of decomposition of forest leaf litter with a mean rate of decomposition of 0.0342 kg/ha/wk (Cuevas & Sajise, 1978). This latter datum was taken under similar conditions as that of the plastic bags. The mean half-life of the plastic materials ranged from 14.60 years

to 20 years. Leaf litters half life was only about 20 weeks or roughly 5 months. (These computations are shown in Table 2).

One particular reason why the plastic bags have very slow rate of decomposition is the paucity of microflora that attack the materials. In the study by Cuevas and Uyenco, (1977), monitoring of decomposition of leaf litters showed that the litters had about 1.3 X 10^3 fungal population/g dry wt of litter on the first week that the litters dropped to the forest floor. A great diversity of mycoflora compose this population with almost all groups of terrestrial fungi represented. In this study of the decomposition of sheets of plastic bags, fungal population was very minimal with only four species isolated even if the plastic sheets were already one month on the forest floor.

CONCLUSION

This study had shown that even the thinnest plastic bags are non biodegradable following the definition of biodegradability as complete decomposition within the lifespan of man. Considering a mean half life of 14.6 years of plastic bags, complete decomposition will take more than 100 years, as compared to leaf litters that remain only on the forest floor for about two years and they are completed decayed. Therefore use of plastic wrappers should be minimized to the least.

As already mentioned in the discussion, composting is an alternative to incineration. To effectively manage the solid wastes in our country, sorting of waste materials at point source into biodegradable and nonbiodegradable should be made mandatory. The biodegradable wastes should be composted.

This study was conducted to prepare fungal inocula suited for rapid decomposition of biodegradable market wastes. Incorporation of fungi that can attack plastic materials is a strategy that will help in the event that not all plastic wrappers are not completely removed from the compost substrates. However, we have to recognize that large quantities of plastic materials in the compost heap will definitely slow down the decomposition process. There is still a need to regulate the amount of plastic that could go to composting even if there is this fungal inocula.

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Interesterification of Coconut and Pilinut Oil Triglycerides By Immobilized Lipase

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ABSTRACT

Immobilization of Candida cylindraceae *lipase, Lipase D from* Rhizopus delemar *and Lipase F "Amano" on three support materials, Amberlite IRA-94s, pig bone particles (150-250 μm) and activated carbon was performed. The performance of the immobilized enzymes on the interesterification of coconut and pili nut oil triglycerides was evaluated by comparing the relative amounts of selected triglyceride fractions of the original and interesterified mixtures. The highest adsorbed protein value per gram of support was obtained with* Candida cylindraceae *lipase on activated carbon. Pre-soaking of the support material appear to lower the protein value except for that on the pig bone support.*

The extent of interesterification was significant for Lipase D immobilized on pre-soaked and dried Amberlite and Lipase F on pre-soaked Amberlite. With Candida cylindraceae *lipase, however, the interesterification profile for all conditions of the experiments did not show a significant difference from that of the original mixture other than a slight increase in the PN48 fraction. Results indicate that pre-soaking of the support material and the amount of adsorbed enzyme affect interesterification performance.*

INTRODUCTION

Modification of fats and oils triglycerides alter some of their physical characteristics such as the melting point, solids profile or crystal habit. One of the technologies for modification is by changing the natural fatty acid distribution in the three positions of the glycerol moiety by enzyme-catalyzed interesterification.

Lipase use for the interesterification of fats and oils is widely investigated because of its specificity which can produce unique triglyceride mixtures. These mixtures are used in food, cosmetics and pharmaceutical industries (Mojovic,1995; Basheer,1995; Macrae,1986).

This research investigated the interesterification of coconut (*Cocos nucifera Linn.)* and pili nut (*Canarium ovatum Engl.)* oils by lipase

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systems. Immobilization of the enzymes on different supports was studied. Enzyme reuse and efficiency is economically promising in tailored fat and specialty oil production for upgrading Philippine oils.

MATERIALS AND METHODS

Materials

The lipases (E.C.3.1.1.3) used were from *Candida cylindraceae* (specific activity 118 units/g); Lipase D derived from *Rhizopus delemar* and Lipase F "Amano 15" from *Rhizopus* strain obtained from the Amano Pharmaceutical Co. Ltd. Lipozyme IM-20, an immobilized lipase preparation was a gift from the Novo Industries. Enzyme supports tested for the lipase immobilization were pig bone particles (150-250 μm) previously prepared in our laboratory ; Amberlite IRA-94s (Rohm and Haas) and Activated carbon (Sigma).

Triglycerides of coconut and pili nut oils were obtained from the Oils and Fats Laboratory, BIOTECH, University of the Philippines at Los Baños, College, Laguna, Philippines.

All other chemicals were reagent grade.

Methods

Lipase Immobilization

One gram of support was added to 10 ml of a solution lipase and 0.05M phosphate buffer (pH 7.7). This was stirred for 1 hr at 0°C and then filtered.

The treatments of the support before immobilization were performed and these are pre-soaking of the support in olive oil for 12 hrs and without pre-soaking.

Two ways of separating the support from the solution after immobilization were carried out and these are washing the support with 50 ml phosphate buffer before vacuum drying and vacuum drying directly without washing. In the case of the olive oil pre-soaked support, washing three times with isooctane after drying was also done.

Protein determination

The support with immobilized lipase was assayed for protein determination using the method of Lowry et.al (1951) with bovine serum albumin as standard.

Interesterification Reaction

The procedure followed was by Sridhar (1991). A 100 mg sample of coconut oil was mixed with 100 mg of pili nut oil after which 3 ml of hexane and 3 g of molecular sieve were added. This mixture was

equilibrated for 1 minute at 40°C and added with 10% (w/w oil) immobilized enzyme. Interesterification was performed on a reciprocal shaking water bath at 40°C for 16 hours.

Samples were withdrawn at 8 and 16 hrs and the enzymatic reaction was halted by filtration of immobilized enzyme through a 0.45 μm Millipore filter. The interesterified oil was collected by evaporation of solvent under a stream of N_2 gas. Interesterification performance of the different immobilized enzymes was evaluated by HPLC areas of the original mixture and interesterified mixtures.

HPLC Separation

High pressure liquid chromatography was performed using Model 556 Gasukuro Kagyo HPLC equipped with an L-6000 Hitachi pump. The column was a stainless steel Novapak C_{18}. Elution was at 0.5 ml/min with acetonitrile:isopropyl alcohol: acetic acid (15:15:1, v/v/v) at room temperature. Peaks were detected by a refractive index detector (Shodex RI SE-51) and processed by a data system. Peaks were identified by comparison with a coconut oil reference material based on retention times of standard triglycerides.

RESULTS AND DISCUSSION

Optimum conditions for immobilization

Previous work in our laboratory showed that the optimum time of immobilization incubation was 60 min and the optimum pH was 7.0. Temperature had little influence on the adsorption of lipase on the support materials. The activity of immobilized lipase at 20°C was 85% of that obtained at 0°C (Negishi, Mukataka et.al, 1989).

The enzyme concentration in an incubated solution is an important factor which affects the amount of enzyme adsorbed, thus, the lipase concentration was varied to determine maximum concentration of adsorbed lipase. For lipase from *Candida cylindraceae* on Amberlite IRA-94s, the amount of adsorbed lipase per gram of support reached a maximum concentration of 5 mg protein/ml of incubated solution (Figure 1). This maximum levelled off at higher concentrations of the enzyme. This concentration therefore was used in subsequent immobilization of other lipases on other supports.

Properties of support materials

The support materials used for immobilization were pig bone particles prepared in our laboratory by cutting pig thigh bone on both ends, autoclave at 120°C for 20 min and ground into particles ranging from 150-250 μm. Amberlite IRA-94 and activated carbon was obtained commercially and had a dp of 450 μm and 100 μm, respectively.

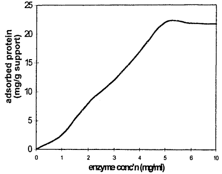

Figure 1. Effect of enzyme concentration on adsorption of lipase. The mixture consisted of 1 gram of support and 10 ml of enzyme solution, shaken for 1 hour at 0 C.

Amberlite which is an ion-exchange resin has no amino functional group and would not work in immobilization with cross linking treatment.

Pig bone particles have been reported to be a useful support material dispersing well both in polar and non-polar solvents. These characteristics present a very important advantage in use as a support material for the immobilization of lipase because lipase is used not only for fat hydrolysis in aqueous or aqueous-organic systems but also for esterification and interesterification in microaqueous systems. It was further shown that pig bone particles has high porosity and this is an additional advantage for the immobilization of enzymes (Mukataka et.al, 1988).

Pig bone particles is composed of about 60% organic and 40% inorganic substances. Most of its organic components is collagen which contains a number of bonding sites.

Effect of solid support pre-treatment

It has been reported (Mukataka,1993)) that pre-soaking the supports was very effective in improving the activity of the immobilized lipase. Vacuum drying of the support materials and the enzyme solution is an advantageous method for adsorbing a large quantity of enzyme in supports. The effect of olive oil pre-soaking and vacuum drying of the support of enzyme was carried out (Table 1-3).

Results (Table 1-3) show the effect of pre-soaking on the amount of adsorbed protein per gram of the support used. It can be seen from Table

1 that with Lipase D (derived from Rhizopus *delemar*), lower protein is adsorbed in pre-soaked material. The difference between pre-soaked and without pre-soaking values are quite significant except in pig bone where protein adsorbed is quite low for this enzyme. With Lipase F (enzyme derived from Rhizopus), the same trend is followed except for pig bone where pre-soaking effected on higher values of adsorbed protein (Table 2). In the case of *Candida cylindraceae* lipase, the trend is the same as in Lipase D, however, adsorbed protein values are higher than those adsorbed with Lipase D and Lipase F. The highest adsorbed protein value was obtained with Candida and activated carbon. Pre-soaking of the support seemed to have lowered the adsorbed protein except for pig bone where pre-soaking has increased adsorbed protein (Table 2). This may have to do with the properties of pig bone material as previously

Table 1. Immobilization of lipase D on different support materials.

Support	Adsorbed Protein (mg/g support)
IRA-94s$_a$	4.8
IRA-94s$_b$	30.24
PB$_a$	2.16
PB$_b$	3.36
AC$_a$	4.56
AC$_b$	14.64

PB- pig bone a- with pre-soaking in olive oil
AC- activated carbon b- without pre-soaking

Table 2. Immobilization of Lipase F on various supports.

Support	Adsorbed Protein (mg/g support)
IRA-94s$_a$	11.6
IRA-94s$_b$	18.56
PB$_a$	8.96
PB$_b$	3.68
AC$_a$	8.24
AC$_b$	15.92

PB- pig bone a- with pre-soaking in olive oil
AC- activated carbon b- without pre-soaking

Table 3. Immobilization of *Candida cylindraceae* Lipase on various supports.

Support	Adsorbed Protein (mg/g support)
IRA-94s$_a$	15.92
IRA-94s$_b$	23.12
PB$_a$	12.8
PB$_b$	20.96
AC$_a$	17.6
AC$_b$	38.24

PB- pig bone a- with pre-soaking in olive oil
AC- activated carbon b- without pre-soaking

mentioned and the enzymes being immobilized. Pig bone particles is quite porous and disperses well in the medium and it contains collagen which has a large number of potential binding sites for enzyme attachment.

Interesterification Performance of Immobilized Lipase

The HPLC analysis of the original mixture of coconut oil and pili nut oil triglycerides showed the presence of 12 major fraction separated based on partition numbers (PN) which is the equivalent carbon number (ECN) of the triglyceride often defined as CN-2n where CN is the carbon number and the n is the number of double bonds. The different triglyceride fractions had partition numbers (PN) which ranged from PN 28 to 52 (Figure 2). Since the objective of this work is to compare the interesterification performance of the lipases immobilized at different conditions, the percent composition of selected peaks such as PN 36 which is the dominating triglyceride in coconut and PN 48 which dominates in pili nut oil were evaluated. Changes in the PN 40 to PN 44 fractions were also evaluated because these fractions could be composed of 1-Lauroyl-2-Oleoyl-3-Palmitoyl glycerol (C_{12}-$C_{18:1}$-C_{16}) and 1,3-Dilauroyl-2-Oleoyl glycerol (C_{12}-$C_{18:1}$-C_{12}) which are the targetted triglycerides of this work. Related research work had shown them to be important in cocoa butter substitutes and other food emulsions (Pham, 1995).

Results after 16 hours of interesterification are shown in Tables 4-6. With Lipase D, interesterification showed significant changes with the enzyme immobilized on Amberlite both with and without pre-soaking and also on pig bone with pre-soaking. It can be seen that using Amberlite with pre-soaking and direct drying, PN 36 decreased by 17% compared with the straight mixed sample; PN 48 also decreased by 29%. On the other hand, PN 40-44 which are the targetted fractions increased from 15.71% to 27.48%. For immobilized Lipase D on Amberlite without pre-soaking,

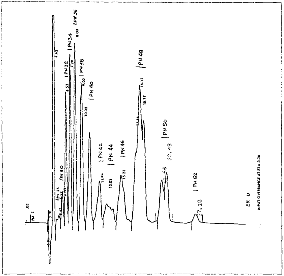

Figure 2. Tryglyceride profile of straight mixed coconut and pili nut oil by HPLC (see Methodology for conditions).

significant change was observed though with lower PN 40-44 values. Therefore, Lipase D on pre-soaked Amberlite without washing and direct drying gave the best interesterification performance.

The results for Lipase F are shown in Table 5. For this lipase, Amberlite gave the best interesterification performance. The PN 36 and PN 48 decreased by 19.69% and 40.25%, respectively. Whereas targetted fractions, PN 40-44, increased from 15.77% to 33.39%. The trend with pre-soaked Amberlite and direct drying gave similar results. This, however, indicates that with Lipase F, pre-soaked Amberlite gave the best interesterification performance which was more than that of Lipase D.

With Candida cylindraceae, however, interesterification profile for all conditions did not show much difference with that of the straight mixed sample except for a slight increase in the PN 48 fractions. This is probably due to the enzyme which is a non-specific one. Candida as reported (Mukataka, 1989) cuts all fatty acids in all three positions of the glycerol moiety.

Table 4. Profile of selected triglycerides of interesterified coconut and pili nut oils using Lipase D immobilized on different supports and varying conditions.

Conditions Enzymes	% composition							
	PN36	PN38	PN40	PN42	PN44	PN46	PN48	PN50
1DA16	10.42	8.65	6.89	5.54	2.96	6.18	30.80	11.50
1DP16	11.04	9.22	7.51	5.98	5.81	5.81	27.56	10.74
1DC16	10.52	8.70	7.40	6.00	5.96	5.96	28.26	10.96
2DA16	8.71	7.74	9.90	7.53	6.85	6.85	21.14	7.78
2DP16	9.75	8.23	8.26	5.83	6.34	6.34	25.70	10.41
2DC16	10.54	8.71	6.70	5.67	6.30	6.30	29.50	11.58
3DA16	12.45	10.52	10.15	6.64	5.01	5.01	19.75	7.88
3DP16	10.09	8.32	7.07	4.82	6.25	6.225	28.53	11.70
3DC16	10.55	8.70	6.80	5.57	6.20	6.20	29.72	11.37

PN - partition number
D - Lipase D
A - Amberlite IRA-94s
P - pig bone
C - activated carbon

1 - pre-soaking, with washing
2 - pre-soaking without washing, direct drying
3 - without pre-soaking, with washing
16 - interesterification time (hrs)

Table 5. Profile of selected triglycerides of interesterified coconut and pili nut oils using Lipase F immobilized on different supports and varying conditions.

Conditions Enzymes	% composition							
	PN36	PN38	PN40	PN42	PN44	PN46	PN48	PN50
1FA16	8.44	8.01	11.29	9.22	12.88	7.37	17.09	6.50
1FP16	9.58	8.24	7.93	5.68	3.82	9.17	27.68	10.52
1FC16	9.54	7.98	6.29	5.29	0.96	8.35	32.13	12.85
2FA16	8.322	7.55	9.97	7.96	11.30	7.17	20.15	7.11
2FP16	9.47	8.12	8.20	5.68	6.220	6.50	26.81	10.57
2FC16	9.82	8.60	7.40	4.60	2.68	7.20	27.65	11.38
3FA16	10.29	8.64	7.17	5.99	3.29	6.33	29.12	11.12
3FP16	10.33	8.72	7.44	4.83	2.58	8.35	29.12	11.08
3FC16	10.42	8.64	7.14	4.64	2.38	7.98	29.65	11.04

PN - partition number
F - Lipase F
A - Amberlite IRA-94s
P - pig bone
C - activated carbon

1 - pre-soaking, with washing
2 - pre-soaking without washing, direct drying
3 - without pre-soaking, with washing
16 - interesterification time (hrs)

Table 6. Profile of selected triglycerides of interesterified coconut and pili nut oils using *Candida cylindraceae* immobilized on different supports and varying conditions.

Conditions / Enzymes	% composition							
	PN36	PN38	PN40	PN42	PN44	PN46	PN48	PN50
1CA16	9.91	8.40	6.79	5.41	2.91	6.31	31.08	12.17
1CP16	10.63	9.00	7.21	5.45	2.96	5.97	30.02	11.36
1CK16	10.42	8.83	7.27	5.91	3.16	6.10	29.55	11.44
2CA16	9.33	8.08	6.87	4.89	4.44	6.82	31.51	12.66
2CP16	9.87	8.39	7.07	4.78	2.322	8.31	30.05	12.27
2CK16	11.58	9.87	7.83	6.03	3.12	5.67	26.79	10.48
3CA16	10.32	8.49	6.89	5.53	2.97	6.10	30.10	12.06
3CP16	10.32	8.68	6.96	5.58	2.89	6.20	30.34	12.03
3CK16	10.02	8.45	6.69	5.48	2.83	6.42	31.26	12.36

PN - partition number
C - Candida cylindraceae
A - Amberlite IRA-94s
P - pig bone
K - activated carbon

1 - pre-soaking, with washing
2 - pre-soaking without washing, direct drying
3 - without pre-soaking, with washing
16 - interesterification time (hrs)

The increase in PN 40-44 fractions of the interesterified samples as compared with the straight mixed samples (Figure 3) indicate that these intermediate fractions may contain the lauric fatty acid from PN 36 and oleic acid from PN 48 in these fractions. Separation of these different triglyceride molecular species using high temperature gas chromatography to verify these fractions is still on-going.

The performance of the laboratory-prepared immobilized lipase gave higher values based on targetted fractions compared to Lipozyme IM-20 (Table 7). The results indicate that pre-soaking of the support material

Table 7. Profile of selected triglycerides of interesterified coconut and pili nut oil using Lipozyme IM-20 and straight-mixed oils.

Conditions	% composition							
	PN36	PN38	PN40	PN42	PN44	PN46	PN48	PN50
interesterified coco-pili	9.87	8.46	9.41	6.41	7.83	6.44	24.33	9.82
straight-mixed	10.51	8.81	6.93	5.77	3.01	6.22	29.81	11.63

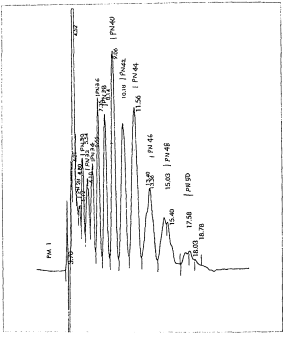

Figure 3. Triglyceride profile of interesterified coconut and pili nut oil by HPLC (see Methodology for conditions).

affects the performance and the amount of adsorbed enzyme. Amberlite IR-94s gave the best interesterification performance with regards to the targetted fractions. However, this is an expensive material. Results of the pig bone support material is not far behind. Thus the preparation of a cheaper immobilized enzyme which could utilize pig bone perhaps may be developed and studied further for interesterification reactions.

ACKNOWLEDGMENT

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Bioactive Furanocoumarin Derivatives from *Ficus Pumila* (Moraceae)

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ABSTRACT

The chloroform extract of Ficus pumila *afforded bergapten and oxypeucedanin hydrate. Their structures were elucidated by extensive 1D and 2D NMR spectroscopy. The compounds were tested for antimicrobial activity against six microorganisms by the agar well method. Bergapten was found to inhibit the growth of* Staphylococcus aureus, Escherichia coli *and* Salmonella typhi, *but was inactive against* Trichophyton mentagrophytes, Mycobacterium pleie *and* Candida albicans. *Oxypeucedanin hydrate inhibited the growth of* Salmonella typhi, *but was found inactive against the other five microorganisms. Both compounds were also tested for antimutagenic activity by the use of the micronucleus test. Results of the study indicated that bergapten reduced the number of micronucleated polychromatic erythrocytes (MPCE) induced by mitomycin C by 44 %, while oxypeucedanin hydrate reduced MPCE by 74%.*

KEYWORDS
antimicrobial, antimutagen, bergapten, creeping fig, *Ficus pumila*, 6,7-furano-5-2,3-dihydroxy-3-methylbutoxy coumarin, 5-methoxy psoralen, Moraceae, oxypeucedanin hydrate

INTRODUCTION

Ficus pumila or creeping fig, an ornamental plant of the family Moraceae, grows vigorously in adobe and concrete walls throughout the Philippines. Its leaves are used in the treatment of dysentery and haematuria, while the juice is employed to treat skin diseases [Quisumbing, 1951]. Previous studies on *Ficus pumila* reported the isolation of 1,4-polyisoprenes [Nwadinigwe, 1988], amyrin acetate, mesoinositol, rutin, sitosterol and taraxenyl acetate [Ts'eng, 1965]. Other species of the genus *Ficus* afforded bergapten, also known as 5-methoxy psoralen [Santos, *et. al.*, 1981; Ahmad, *et. al*, 1976; Abu-Mustafa, *et. al*, 1978; El-Gamal, *et. al.*, 1973; Harkar, *et. al.*, 1984; Beir, *et. al.*, 1994] which is of relevance to our present report.

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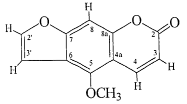
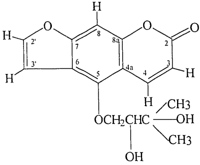
In an earlier study, bergapten was reported to have allelopathic activity *in vitro* on radish germination and radicle growth [Aliotta, *et. al.*, 1994]. It was also found to decrease the relative growth rate and relative consumption rate of the insect, *Spodoptera littoralis* [Hadasek, *et. al.*, 1994]. In another study, bergapten was reported to be an ingredient in several suntan preparations which provide stimulus for melanin deposition [Ashwood-Smith, *et. al.*, 1980]. It was also found to have mutagenic and clastogenic properties when exposed to UV light [Ashwood-Smith, *et. al.*, 1980]. Previous studies reported that it is a potent photosensitizing agent used to treat skin depigmentation, psoriasis, photodermatitis and eczema [Aliotta, *et. al.*, 1994; Diawara, *et. al.*, 1995].

We now report the isolation, structure elucidation and bioassay of two compounds : bergapten (**1**) and oxypeucedanin hydrate (**2**) from the chloroform extract of *Ficus pumila*. This is the first report on the isolation of **1** from *Ficus pumila* and **2** for the genus *Ficus*.



Compound **1**



Compound **2**

RESULTS AND DISCUSSION

Compounds 1 and 2 were isolated from the chloroform extract of *F. pumila*. Their structures were elucidated by extensive 1D and 2D NMR spectroscopy. The antimicrobial activity of 1 and 2 was determined by the agar well method, while their antimutagenecity potential was evaluated by the micronucleus test.

The ^1H NMR spectrum of 1 indicated resonances for aromatic protons at δ 8.14 (1H, d, J= 8.9 Hz), δ 6.26 (1H, d, J = 8.9 Hz), δ 7.12 (1H, s), δ7.59 (1H, d, J = 1.8 Hz) and δ 7.02 (1H, d, J =2.4 Hz) and a methoxy group at δ 4.27 (3H, s). The aromatic system was supported by the FT-IR spectrum which showed aromatic C-H stretches at 3162, 3132 and 3087 cm^{-1}, C=C stretches at 1625 and 1580 cm^{-1} and aromatic CH out of plane bending at 847 and 776 cm^{-1}. The COSY spectrum of 1 indicated two isolated spin systems as follows: the proton at δ 8.14 was coupled to the hydrogen at δ 6.26 by 9.8 Hz and the proton at δ 7.59 was coupled to the hydrogen at δ 7.02 by 2.4 Hz. The large coupling constant of 9.8 Hz was attributed to a six membered aromatic system with protons oriented *cis* to each other, while the small coupling of 2.4 Hz indicated a furan with *cis* protons [Silverstein, *et. al.*, 1981].

The ^{13}C and DEPT NMR spectra of 1 showed the presence of twelve carbons with the following functionalites: five protonated carbons and five non-protonated carbons in the aromatic region, a carbonyl and a methoxy carbon (Table 1). Among the ten aromatic carbons, four are oxygenated as indicated by their deshielded resonances at δ 158.4, 152.7, 149.6 and 144.8. To determine which hydrogens were directly bonded to carbons, the ^{13}C-detected heteronuclear shift correlation (CHSHF) was obtained (Table 1). It indicated that the protons at δ 6.26, 8.14, 7.12, 7.59 and 7.02 were bonded to the aromatic carbons at δ 112.6, 139.3, 93.8, 144.8, and 105.1, respectively, while the methyl protons at δ 4.27 (3H, s) were bonded to the carbon at δ 60.1. The ^{13}C-^1H connectivities were verified by the ^1H-detected Heteronuclear Multibond Shift Correlation (HMBC) which determined the long-range correlations between ^1H and ^{13}C nuclei two to three bonds away (Table 1). Thus, the protons at δ 6.26 and 8.14 were long-range correlated to the carbon at δ 161.2; the protons at δ 7.12 and 8.14 were long-range correlated to the carbon at δ 152.7; the protons at δ 7.12 and 6.26 were correlated to the carbon at δ 106.5; the protons at δ 4.27 and 8.14 were correlated to the carbon at δ 149.6; the proton at δ 7.12 was correlated to the carbons at δ 158.4 and 112.7.

Table 1. ^1H, ^{13}C, CHSHF and HMBC spectral data of compound 1 in $CDCl_3$.

^{13}C-DEPT type	^{13}C shift, d	*Short Range* heteronuclear expt. (CHSHF) ^{1}H shift, d	*Long Range* heteronuclear expt. (HMBC)
C2	161.2		H4, H3
C3	112.6	6.26 (1H, d, 9.8 Hz)	no crosspeak
C4	139.3	8.14 (1H, d, 9.8 Hz)	H3
C4a	106.5		H8, H3
C5	149.6		$-OCH_3$, H4
C6	112.7		H2', H8, H3'
C7	158.4		H2', H8, H3'
C8	93.8	7.12 (1H, s)	no crosspeak
C8a	152.7		H4, H8
furano C2'	144.8	7.59 (1H, d, 2.4 Hz)	H3'
furano C3'	105.1	7.02 (1H, d, 2.4 Hz)	H3'
$-OCH_3$	60.1	4.27 (3H, s)	no crosspeak

The structure of **1** was supported by the FT-IR absorptions at 1731 cm $^{-1}$ (C=O stretch) and at 1222, 1157 and 1123 cm $^{-1}$ (C-O-C stretches). It was further supported by the UV spectrum which showed λ_{max} = 310 nm, indicative of a β-monosubstituted unsaturated ester [8]. Literature search revealed that **1** is bergapten as evidenced by similar ^{1}H and ^{13}C NMR spectral data and identical melting points [Beir, *et. al.*, 1994].

Comparison of the ^{1}H NMR spectra of **1** and **2** (Table 2) indicated that they are similar. They differ only in the substituents attached to C5. Compound **1** had a methoxy group, while **2** had a 2,3,-dihydroxy-3-

Table 2. Comparison of 400 MHz ^{1}H-NMR spectral data of comounds 1 and 2 in $CDCl_3$.

^{1}H shift, δ Compound 1	1H shift, δ Compound 2
6.26 (1H, d, J=9.9 Hz)	6.28 (1H, d, J=9.8 Hz)
8.14 (1H, d, J=9.8 Hz)	8.16 (1H, d, J=9.8 Hz)
7.12 (1H, s)	7.16 (1H, s)
7.59 (1H, d, J=2.4 Hz)	7.61 (1H, d, J=2.4 Hz)
7.02 (1H, dd, J=2.4, 0.8 Hz)	6.99 (1H, d, J=2.4 Hz)
4.27 (3H, s)	
	4.54, 4.45 (2H, m)
	3.92 (1H, s, br)
	1.32 (3H, s)
	1.37 (3H, s)
	2.91, 2.21 (2OH)

methylbutoxy attached to C5. Thus, the resonance at δ 4.27 which was assigned to a methoxy group in **1** was no longer found in **2**. In place of the methoxy group were the resonances attributed to 2,3-dihydroxy-3-methylbutoxy at δ 4.54 (1H, m), δ 4.45 (1H, m), δ 3.92 (1H, s, br), δ 1.32 (3H, s), δ 1.37 (3H, s), δ 2.91 (OH, s, br) and δ 2.21 (OH, s, br). The presence of OH's in **2** was supported by the FT-IR spectrum which showed a broad OH stretch at 3448 cm^{-1}.

The ^{13}C and DEPT NMR spectra of **2** (Table 3) showed the loss of the methoxy carbon at δ60.1 and the appearance of resonances attributed to 2,3-dihydroxy-3-methylbutoxy at δ 74.5, 76.5, 71.9, 26.7 and 25.1. The carbons at δ 106.5 (C4a), δ 93.8 (C8) and δ 112.7 (C6) were deshielded to δ 107.3, 94.8 and 114.3, respectively, while the carbon at δ 149.6 (C5) was shielded to δ 148.5 as a result of the change in substituent.

Table 3. ^{13}C and DEPT spectral data of compounds 1 and 2.

Carbon	^{13}C shift, δ Compound 2	^{13}C shift, δ Compound 1
C2	161.1	161.2
C3	113.0	112.6
C4	139.1	139.3
C4a	107.3	106.5
C5	148.5	149.6
C6	114.3	112.7
C7	158.1	158.4
C8	94.8	93.8
C8a	152.5	152.7
furano C2'	145.3	144.8
furano C3'	104.7	105.1
side-chain		
C1"	76.5	
C2"	74.5	
C3"	71.7	
C4"	26.7	
C5"	25.1	
methoxy C1"		60.1

The structure of **2** was further supported by the COSY, CHSHF and HMBC NMR spectra. In addition to the two isolated spin systems in **1**, the COSY of **2** indicated coupling between the carbinyl protons at δ 4.45 and 4.54 and the carbinyl proton at δ 3.92. The CHSHF spectral data of **2** (Table 4) indicated additional ^{13}C-^{1}H short range correlations as follows: the methylene protons at δ 4.45 and 4.54 were bonded to the oxygenated carbon at δ 76.5, while the hydrogen at δ 3.92 was bonded to the oxygenated carbon at δ 74.5. The methyl protons at δ 1.37 and 1.32 were bonded to the carbons at δ 26.7 and 25.1, respectively. Results of HMBC

(Table 4) confirmed the structure of 2. Thus, additional long-range correlations were deduced for the 2,3-dihydroxy-3-methylbutoxy substituent. The methyl carbon at δ 26.7 is long-range correlated to the methyl protons at δ 1.32 and the hydroxyl proton at δ 2.92, while the other methyl carbon at δ 25.1 was correlated to the methyl protons at δ 1.37. The nonprotonated carbinyl carbon at δ 71.7 was long-range correlated to the methyl protons at δ 1.32 and 1.37, while the carbinyl carbon at δ 76.5 was correlated to the hydroxyl proton at δ 2.21.

Table 4. ^{1}H, ^{13}C, CHSHF and HMBC spectral data of compound 2 in $CDCl_3$.

Carbon	^{13}C shift, δ	Short Range heteronuclear expt (CHSHF) ^{1}H shift, δ	Long Range heteronuclear expt (HMBC)
C2	161.1		H3, H4
C3	113.0	6.28 (1H, d, 9.8 Hz)	H4
C4	139.1	8.16 (1H, d, 9.8 Hz)	*no crosspeaks*
C4a	107.3		H8, H3
C5	148.5		H4
C6	114.3		H2', H8, H3'
C7	158.1		H2', H3'
C8	94.8	7.16 (1H, s)	*no crosspeaks*
C8a	152.5		H4, H8
furano C2'	145.3	7.61 (1H, d, 2.2 Hz)	H3'
furano C3'	104.7	6.99 (1H, s)	H2'
C1"	74.5	4.45, 4.54 (2H, m)	
C2"	76.5	3.92 (s, br)	2" OH
C3"	71.7		4", 5"
C4"	26.7	1.37 (3H, s)	5", 3" OH
C5"	25.1	1.32 (3H, s)	4"

Extensive literature search revealed that 2 is oxypeucedanin hydrate or 6,7-furano-5-2,3-dihydroxy-3-methylbutoxy coumarin. Confirmatory evidences were the ^{1}H and ^{13}C NMR spectral data of 2 and oxypeucedanin hydrate [Beir, *et. al.*, 1994]. The spectra of 2 and oxypeucedanin hydrate matched in all essential respects. In addition, the melting points of the two compounds are similar [Beir, *et. al.*, 1994]. Although 2 was previously reported from *Peucedanin ostruthium* and other species of *Phebalium* plants [Beir, *et. al.*, 1994], this is the first report on its isolation from the genus *Ficus*.

A previous study [Quisumbing, 1951] reported that the juice of the plant was used for the treatment of skin diseases, hence a potential source of antimicrobial compounds. Antimicrobial tests of 1 and 2 using the same six target microorganisms were conducted. Results of the antimicrobial study on 1 (Table 5) indicated inhibitions against *S. aureus*, *E. coli* and *S. typhi* at concentrations of 58

Table 5. Antimicrobial test results on 1 and 2.

Sample	Concn. (µg)	*Staphylococcus aureus*		*Escherichia coli*		*Salmonella typhi*		*Candida albicans*		*Trichophyton mentagrophytes*		*Mycobacterium pleie*	
		C.Z.* (mm)	A.I.	C.Z.* (mm)	A.I. •	C.Z.* (mm)	A.I. •	C.Z.* (mm)	A.I. •	C.Z.* (mm)	A.I. •	C.Z.* (mm)	A.I. •
Compound 1	29	-----	0	-----	0	-----	0	-----	0	-----	0	-----	0
	58	13	0.3	12	0.2	14	0.4	-----	0	-----	0	-----	0
	87	16	0.6	14	0.4	19	0.9	-----	0	-----	0	-----	0
Compound 2	22	-----	0	-----	0	-----	0	-----	0	-----	0	-----	0
	44	-----	0	-----	0	14	0.4	-----	0	-----	0	-----	0
	65	-----	0	-----	0	16	0.6	-----	0	-----	0	-----	0
	78	-----	0	-----	0	18	0.8	12	0.2	-----	0	-----	0
	104	-----	0	-----	0	22	1.2	12	0.2	-----	0	-----	0
	130	-----	0	-----	0	26	1.6	12	0.2	-----	0	-----	0
Standard Antibiotic	30	33	2.3	24	1.4		1.3	24	1.4	68	5.8		
		Chloramphenicol		Tetracycline		Chloramphenicol		Clotrimazole		Clotrimazole		NT•	

*Clearing Zone
•Activity Index
•Not tested - std. antibiotic not available

and 82 μg. On the other hand, **2** showed inhibition against *S. typhi* at concentrations of 130, 104, 78, 65 and 44 μg with activity indices of 1.6, 1.2, 0.8, 0.6 and 0.4, respectively. However, compared to standard antibiotics, **1** and **2** are less active.

In view of the aforementioned studies on the mutagenicity of **1** when exposed to UV light [Ashwood-Smith, *et. al.*, 1980], the compound was tested for its effect on the formation of micronucleated polychromatic erythrocytes (MPCE) induced by a known mutagen, mitomycin C. Results of the study (Table 6) indicated that at a dosage of 8 mg/kg mouse, **1** reduced the number of MPCE induced by mitomycin C by 44%.

Table 6. Effects of 1 and 2 on the formation of micronucleated polychromatic erythrocytes induced by mitomycin C.

Sample	Ave. no. of MPCE/1000 PCE ±	% Reduction in MPCE
1	4.58 ± 1.16 s	44
2	2.11 ± 0.54	74
CONTROL	8.17 ± 1.47	

*Average of 15 slides

Statistical analysis using t-test showed that there is a significant decrease of MPCE at $\alpha = 0.01$. Thus, in the absence of UV light, **1** is an antimutagen. Results of the study on **2** showed 74 % reduction in MPCE at the same dosage. Statistical analysis using t- test showed that there is a significant decrease in MPCE at $\alpha = 0.01$. Thus, **2** has a higher antimutagenic activity than **1**.

CONCLUSION

The chloroform extract of the air dried leaves of *F. pumila* afforded two furanocoumarin derivatives: bergapten and oxypeucedanin hydrate. Bergapten was found to inhibit the growth of *E. coli* (gram negative bacteria), *S. aureus* (gram positive bacteria) and *S.typhi* (rod shaped organism that cause typhoid fever), while oxypeucedanin hydrate specifically inhibited the growth of *S. typhi*. Thus, bergapten and oxypeucedanin hydrate can be used to prevent the proliferation of the tissues of any infection caused by *S. typhi*. Oxypeucedanin hydrate was found to be a better antimutagen than bergapten.

EXPERIMENTAL

General:

The identity of **1** and **2** was established by spectroscopic methods. NMR spectra were recorded with the use of a JEOL Lambda Fourier Transform 400 MHz NMR. All spectra were recorded in $CDCl_3$. The IR analysis

was done using a Perkin Elmer 1600 Fourier Transform IR spectrometer and the UV spectrum was obtained from a HP 8452A diode array spectrometer. Fractions were monitored by TLC and spots were visualized by spraying with vanillin/H_2SO_4, followed by warming. The melting point was obtained by the use of a Fischer-Johns melting point apparatus. The number of MPCE was determined by the use of a Zeiss microscope.

Sample Collection

The sample was collected in Kalookan City in December 1995. It was identified at the National Museum as *Ficus pumila* and vouchers have been deposited at the Philippine National Herbarium (PNH # 14540).

Isolation

Air dried leaves (889 g) were soaked in 3.5 L chloroform, then filtered. The filtrate was concentrated under vacuum to afford a crude extract which was treated with 4% aqueous $Pb(OAc)_2$ solution to precipitate the pigments. The treated extract (12.8 grams) was subjected to gravity column chromatography packed with silica gel (60-230 mesh) and eluted with increasing proportions of acetone in chloroform (10% increment). The fractions eluted with chloroform and 10% acetone in chloroform were rechromatographed in chloroform to afford **1** (25 mg, colorless crystal, mp = 179°C) after recrystallization from diethyl ether. The fraction eluted with 30% acetone in chloroform was rechromatographed in 20% acetone in chloroform to afford **2** (10 mg, colorless crystals, mp = 134°C) after recrystallization from diethyl ether.

Antimicrobial Test

Microbial suspension containing approximately 10^7 cells/mL was prepared for each test organism for 24-hour agar culture using 0.1% peptone water. One-tenth (0.1 mL) of the bacterial suspension was transferred into pre-poured 30 mL deep nutrient agar plate, the yeast suspension poured into glucose yeast peptone agar plate, while the fungal suspension on potato dextrose agar plate. About 5 mL of the corresponding melted agar cooled to about 45°C was immediately poured into the plate. The plate was swirled to distribute the microbial cells evenly on the plate. After the overlay agar has solidified, three 1-cm diameter holes were bored form equidistant points using a sterile cork borer.

One-tenth (0.1 mL) portions of the extract were placed in duplicate holes per organism. A similar volume of the acetone solvent and of the corresponding antibiotic for each test organism was placed in the remaining two wells on the plate. Plates were incubated at room temperature to prevent evaporation of liquid on the petri lid that may cause interference in the distribution of organisms on the surface. Bacterial and yeast plates were scored after 24 h, while the mold plate was read after 72 h. Zones of inhibitions were measured in millimeters (mm),

the average for each sample was taken and the antimicrobial activity index (AI) was computed as the zone of inhibition of the sample minus the diameter of the hole divided by the diameter of the hole.

Antimutagenicity Test: The test compounds (8 mg/kg mouse) dissolved in DMSO (7.5 mL/kg mouse, solvent control) were administered simultaneously with mitomycin C (2.75 mg/kg mouse, positive control) to mice of the Swiss strain (source: DOST). For the control, only mitomycin C and DMSO were administered orally to the mice. Five mice were tested for each compound and control. The second administration was carried out after 24 h. Six hours after the second administration, the mice were sacrificed by dislocation of the neck. Blood from the bone marrow was smeared on slides (three per mouse). The slides were stained with May Grunwald and Giemsa solutions [Schmid, 1976]. The numbers of MPCE/1000 PCE were counted by the use of a high power microscope.

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Insecticidally Active Sesquiterpene Furan from *Bontia Daphnoides L.*

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ABSTRACT

Activity guided isolation of the crude ethanol extract of the leaves and stems of Bontia daphnoides using adult Cylas formicarius as the test organism revealed that the most insecticidally active compound was the sesquiterpene furan, epingaione. This compound was previously isolated and characterized by Chinnock et al. (1987). The 72 hour Lc_{50} values (μg/insect) found for the furan was 20.80 (17.34 - 24.52), while the values obtained for a nicotine rich extract of tobacco and a commercial organophosphate insecticide, dimethoate were 32.00 (28.15 - 40.32) and 9.54 (8.40 - 10.62) μg/insect, respectively.

INTRODUCTION

The long term use and misuse of conventional synthetic insecticides (organochlorines, organophosphates and carbamates) has created many ecological and environmental problems (Brown, 1978; Flickinger *et al.*, 1986; Stromborg, 1986). Chief among these problems include: induction of resistance in several pest species (Georghiou and Melon, 1982), toxicity to non-target organisms including man and the natural enemies of various pest species (Reid, 1987; Georghiou and Melon, 1982), contamination of several water bodies (National Research Council of Canada, 1975; Williams and Chow, 1993) and high levels of residues in agricultural food products (Thomson and Abbott, 1967; Vettorazzi and Miles-Vettorazzi, 1975; Flickinger *et al.*, 1986). Thus, there is an urgent need to find safer pesticides for use in present day pest management programmes. One source of these safer pesticides is the secondary metabolites (natural products) isolated from plants (Williams, et al., 1989). These compounds have demonstrated a diverse array of biological actions on arthropod species which include disruption of cytochrome P450 activities by monoterpenes (Brattsten, 1982); retardation of corpora allata development by the precocenes (Bowers, 1982) and inhibition of neuroendocrine processes, protein and lipid metabolisms by several limonoids e.g. the azadirachtins (Rembold *et al.*, 1984; Sieber, *et al.*, 1984; Bidmon *et al.*, 1987; Dorn *et al.*, 1987; Wilps, *et al.*, 1992).

Williams (1992) demonstrated that extracts from the leaves and stems of *Bontia daphnoides* were effective in killing *Tribolium confusum*. These findings were confirmed in a later study by Williams and Mansingh

(1993). However, the major insecticidally active compound (s) was not elucidated from the extract. Thus, in the present study an attempt was made in isolating and elucidating the active compound.

MATERIALS AND METHODS

Preparation of crude extracts and fraction from B. daphnoides

Leaves and stems of *B. daphnoides* were air dried in the laboratory for 5 days. Dried plant material weighing 400 g was then pulverized and extracted with 8.0 L of 95 % ethanol for 120 hrs. The resulting crude extract was then concentrated *in vacuo* to 20 mL using a rotary evaporator. To the concentrated crude extract 200 mL of hexane and 200 mL of a methanol-water (10: 1; v/v) mixture were added for separating the extract into non-polar (hexane) and polar (water-methanol) fractions for bioassay.

Separation and isolation of the active compound in the hexane fraction

A twenty gram portion of the hexane fraction was redissolved in hexane and separated using a solvent system of 12.0 % ethyl acetate in petroleum ether (30°C to 40°C) on a flash glass column. The glass column of 75.0 cm length and dia of 2.5 cm was packed with kieselgel 60 (particle size = 0.07 mm) and pressurized with air at 1 bar/min during the separation process.

Aliquots of 20 mL were collected and assayed for insecticidal activities as stated below. Fractions 19 - 26 showed significant toxicity to the weevils. These fractions were then pooled and purified by thin layer chromatography using 10.0% ethyl acetate in petroleum ether.

Insecticidal assay

Test insects

Adult *Cylas formicarius elegantulus* Summer (Coleoptera: Curculionidae) (a Sweet potato weevil) were cultured on *Ipomea* spp (Sweet potatoes) in glass aquaria at 37°C and 78 - 80% RH in the laboratory. Insects weighing 40 $\pm$ 1.50 mg were used in the assays.

Bioassays

Thirty adult *C. formicarius* were topically treated in three replicates of 10 each on the ventral surface of their abdomen with doses ranging from 1.0 - 50 μg of the crude ethanol extract in acetone. A similar dose range was used for assessing the toxicity of the hexane, ethyl acetate, methanol fractions and pure compounds. For comparative analyses the toxic action of the active compound isolated from *B. daphnoides* was compared to the toxicity of a nicotine rich extract of tobacco and dimethoate a commercial pesticide. The control insects were treated with 6 μL of acetone only.

The treated insects were fed potato disc of 2.0 cm^3 and the number of insects dead after 72 hours recorded. These mortality data were then subjected to Probit Analysis according to Busvine (1972) for calculating the concentration of extracts, compounds, or pesticide required for killing 50% of the test insects (Lc_{50} values).

Comparison of the toxicity of the sesquiterpene furan with two commercial pesticidal agents

1. **Dimethoate:** O,O-dimethyl-S-methylcarbamoylmethyl phosohorodithioate: This commercial insecticide was used to compare the activity of the sesquiterpene furan isolated from *B. daphnoides*.

2. **Crude tobacco alkaloid extract:** The pest management potential of tobacco extracts is known from as early as the 17th century (Rao and Chakarborty, 1982). Thus, a crude alkaloid extract of tobacco was prepared for comparing the toxicity of the fractions and pure compounds isolated from *B. daphnoides*. Briefly, 620 g of dried leaves and stems of tobacco were milled into small pieces and defatted with 0.5 L of petroleum ether. The defatted material was then extracted in 4% aqueous sodium hydroxide solution for 72 hrs to allow the formation of the free bases of the alkaloids. The brown solution produced a filtrate which was extracted with chloroform. The resulting extract was concentrated *in vacuo* using a rotary evaporator to produce 12.4 g crude alkaloid extract.

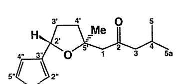
RESULTS AND DISCUSSION

Structural elucidation of the active compound

The major insecticidal compound was resolved at an Rf value of 0.37. Analyses of the following spectral data: infrared, ^{13}C-NMR, ^{1}H-NMR (300 mHz) and mass spectrum (IE) revealed that the above compound was Epingaione, a sesquiterpene furan with a yield of 1.8 % of the dried plant material. Chinnock *et al.*, (1987) isolated this compound from *B. daphnoides* and determined its complete stereochemistry as (-)-Epingaione = [(-)-(1S, S4)-beta-(4,8-dimethyl-6-oxo-1-, 4-epoxynonyl furan]. The major infrared peaks (neat on NaCl disc) found were: 1680 (C==C) cm^{-1}; 1700 (C==O) cm^{-1} and 3120, 1507 and 866 cm^{-1} for the furan ring.

The ^{13}C NMR data (ppm) found were:

Carbons	ppm value
1	53.7
2	209.4
3	53.7
4	24.7
5	22.44
5a-Me	22.44
2'	73.51
3'	33.38
4'	37.16
5'-Me	27.85
2"	143.43
3"	127.50
4"	108.72
5"	139.34



The above mentioned ^{13}C data were consistent with those published by Chinnock *et al.*, (1987) for the same compound.

GC-MS analyses were performed on a VARIANT 3700 M80 - 44X machine using a DB1 capillary column at a temperature programme of 50 - 250°C at 10°C/min: Rt = 5.0 min. The mass spectral data m/z (relative intensity) found were: 250 $[m]^+$ (5), 150 (62), 110 (100), 95 (22), 85 (75), 83 (30), 57 (84) (Fig. 1.). The base peak (peak of 100% intensity) occurred at m/z of 110 and is attributed to the formation of the furan alcohol (Fig. 1). The molecular weight of the other fragments formed from the furan upon ionization are also shown.

The proton NMR (250 mHz, CCl_4) data found were: δ 0.95 (6H, d, J = 6.6 Hz, Me-5, 5a-Me), 1.27 (3H, s, 5'-Me), 1.87 (2H,m), 1.87 (2H, m H_2-3' and H_2-4'), 2.59 (1H, s, H_2-1), 2.4 (1H, s, H-4), 2.75(2H, s, H_2-1), 4.9 (1H, m, H_2-2'), 2.15 (1H, m, H-4) and 7.27 and 7.30 (2H, m, H-2" and H-5"). These proton data were also consistent with those reported by Chinnock *et al.*, (1987); Hamilton *et al.*, (1973).

Insecticidal studies

The data obtained on the insecticidal activity of the different extracts of *B. daphnoides* revealed that the major toxic compound was localized in the hexane fraction. Thus the Lc_{50} value calculated for the crude extract was 31.23 μg/insect while that obtained for its hexane counterpart was 17.40 μg. The hexane fraction was followed in potency by the ethyl acetate and methanol fractions with Lc_{50} values of 63.22 and 150 μg/insect respectively (Table 1). Further analyses of the data presented in Table 1

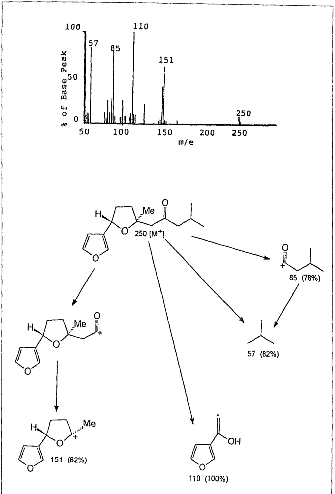


Figure 1. Mass spectrum of epingaione and associated fragments.

Table 1. Seventy two toxicity data of the crude ethanol extract and fractions of *Bontia daphnoides* and the alkaloid fraction of tobacco and dimethoate against *Cylas formicarius*.

Crude extract fractions and compound	72 hr LC_{50} values (mg/insects) with Fiducial limits
Crude extract	31.23 (28.02 - 37.33)
Fraction	
Hexane	17.40 (13.30 - 21.82)
Ethyl acetate	63.22 (55.41 - 79.37)
Methanol	150.00 (132.52 - 165.81)
Pure furan: Epingaione	20.80 (17.34 - 24.52)
Commercial pesticides	
Dimethoate	9.50 (8.42 - 10.64)
Nicotine rich extract	32.00 (28.00 - 40.22)

revealed that there were no significant differences between the toxicity of the furan and the hexane fraction which suggest that the furan is the major toxic element in the fraction.

Crude nicotine extracts of tobacco are well known for their pest control potential globally (Rao and Chakarborty, 1982). Thus, it is interesting to note that the furan isolated from *B. daphnoides* was more toxic to the insects than the tobacco extract and was 50% less effective than the organophosphate insecticide dimethoate (Table 1). These correlations would suggest that this compound could be a potential agent for the management of insect pests.

CONCLUSION

The insecticidal activity demonstrated by *Bontia daphnoides against C. formicariius* is due mainly to the presence of the sesquiterpene furan epingaione.

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Genotoxicity of Pigments from Seeds of *Bixa Orellana* L. (Atsuete) II. Determined by Lethal Test

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ABSTRACT

Pigments from dried atsuete (Bixa orellana L.) seeds were extracted by using chloroform AR grade. The chloroform was evaporated to dryness and the residue was designated crude atsuete-chloroform extract (CE). CE was treated with petroleum ether AR grade. The residue was dried and designated as CE residue-petroleum ether extract (BE). BE was subjected to TLC. Male Namru strain (NMR) mice were given varying concentrations of BE and CE, and then mated with untreated females.

More or less the same number of females mated with males given varying concentrations of BE became pregnant. However, more dead implants were observed in female mice mated with males given BE than those of the control indicating that BE is genotoxic. There were 2.1, 8.6, 18.3, and 20.4% dead implants in female mice mated with males given distilled water, DMSO, 200 mg BE per 1 kg body weight and 400 mg BE per 1 kg body weight, respectively. The percentage of female mice that became pregnant and had dead implants were 14.2, 33.0, 83.3, and 100% for female mice mated with males given distilled water, DMSO, 200 mg BE per 1 kg body weight, and 400 mg BE per 1 kg body weight, respectively.

Pregnancy in female mice mated to males given DMSO, 200 mg CE per 1 kg body weight, and 400 mg CE per 1 kg body weight were 50, 75, and 25%, respectively. There were 5.7, 9.2, and 26.9% dead implants in female mice mated with males given DMSO, 200 mg CE per 1 kg body weight, and 400 mg CE per 1 kg body weight, respectively. CE, like BE, is genotoxic.

INTRODUCTION

Atsuete is used in traditional Filipino cooking as a coloring agent. It is used also in coloring butter, margarine, cheese, beverages, meat and fish products. The atsuete dye, known also as annatto dye, is extracted from the outer covering of the seeds of *Bixa orellana* L. of the Family Bixaceae.

Banzon and Arañez (1984) reported that dried crude atsuete extract prepared by using chloroform as the extracting liquid is mutagenic as determined by the *in vivo* Micronucleus Test and Drosophila Test. The

crude atsuete-chloroform extract produced a significant increase in the micronucleated polychromatic erythrocytes in mice that were given 125 mg and 300 mg crude extract in DMSO per 1 kg body weight. When crude atsuete-chloroform extract was treated with petroleum ether, the residue also produced micronucleated polychromatic erythrocytes but the frequency was not significantly different from the control. The number of micronucleated polychromatic erythrocytes produced by petroleum ether extract was significantly higher than the control.

The *Drosophila* Test (Banzon and Arañez, 1984) produced the same results as the Micronucleus Test. The crude atsuete-chloroform extract and the petroleum ether extract produced significant increases in the number of flies exhibiting wing abnormalities. The crude extract residue after treatment with petroleum ether also produced wing abnormalities but the frequency was not significantly different from the control.

Banzon and Arañez (1984) reported that the *in vitro* bacterial test using Salmonella typhimurium TA 1535, TA 1537, TA 98, and TA 100 did not produce significant increases in the number of revertant colonies in the three extracts tested. The mutagenic agent in atsuete pigment appeared to be a pro-mutagen (Banzon and Arañez, 1984). No microsomal enzyme was used in the above tests.

Arañez and Rubio (1996), using the *Allium* Test, observed that the crude atsuete-chloroform extract (CE) and pure petroleum ether extract (PE) were genotoxic at 1.5%, 3.0%, and 4.5% (w/v) as shown by the significantly high number of mitotic aberrations in the form of anaphase bridges, ring chromosomes, precocious chromosomes, and disoriented chromosomes. Mitotic aberrations in cells exposed to CE residue-petroleum ether extract (BE) treatments were not statistically different from the control.

Before BE could be declared safely as non-genotoxic, different tests are needed to confirm the results. The Dominant Lethal Test may confirm the results obtained from other tests. At the same time it indicates the ability of the agent to penetrate gonadal tissue and produced chromosomal damage (Green *et al.*, 1985). Green *et al.*, (1985) mentioned also that the Dominant Lethal Assay is one of the few tests in which the mutagenic potential of an agent is examined directly in gametic tissue in an intact mammal. It is also of importance in mutagenicity testing since it provides an evidence that genetic damage to somatic cells is reflective of similar damage to germ cells. The Dominant Lethal Assay has also been used to screen for agents that produce chromosomal breakage in gametic cells (Green *et al.*, 1985).

The objective of this study is to confirm the non-genotoxicity of the atsuete pigment from the crude extract residue after treatment with petroleum ether (BE).

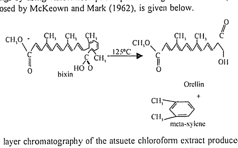
REVIEW OF LITERATURE

The atsuete dye has two coloring matters, namely bixin and orellin (Tanchico and West, 1936). Bixin is the principal pigment of atsuete (McKeown and Mark, 1962). It is carotenoid in nature and constitutes about 55 to 60% of the total pigment (Gregory, 1939). It occurs also as brick red needle-like crystals, and is soluble in water, ethyl alcohol, methyl alcohol and chloroform. However, it is insoluble in petroleum ether (Batoon, 1978). The structural formula of bixin (Kuhn and Winterstein, 1928) is given below.

$$H_3COOC\text{-}CH{=}CH\text{-}\underset{|}{\overset{CH_3}{C}}{=}CH\text{-}CH{=}CH\text{-}\overset{CH_3}{C}{=}CH\text{-}CH{=}CH\text{-}CH{=}\overset{CH_3}{C}\text{-}CH{=}CH\text{-}CH{=}\overset{CH_3}{C}\text{-}CH{=}CHCOOH$$

The cis-form is unstable and is transformed by heat to the stable trans-isomer (McKeown and Mark, 1962). The molecular weight of bixin is 394.49. It is soluble in alkalies and is precipitated by acids (Devon and Scott, 1972).

Orellin constitutes the lesser component of the annatto extract (McKeown and Mark, 1962). It is a yellow-orange wax-like liquid, soluble in ethyl alcohol, methyl alcohol, petroleum ether, and chloroform, but insoluble in water (Batoon, 1978). McKeown and Mark (1962) were the first to elucidate on the possible structure of orellin. Iversen and Lam (1952) and McKeown and Mark (1962) observed that bixin is degraded by heat by affecting the double bonds. McKeown and Mark proposed that bixin undergoes degradation when subjected to high temperature extraction forming a yellow pigment plus meta-xylene. The pigment produced is presumed to be orellin, the monomethyl ester of the unsymmetrical tetrahexane-dioic acid. Osteraas and Olsen (1967) confirmed these findings by using visible absorption spectra. Degradation of bixin, as proposed by McKeown and Mark (1962), is given below.



Thin layer chromatography of the atsuete chloroform extract produced five spots of which one major spot could be cis-bixin, one minor spot could be orellin, while the other spots are likely to be the degradation products of cis-bixin (Tong, 1984).

Annatto was reported to be rich in vitamin A. When rats were fed with vitamin A-free diet containing crude annatto extract, the growth rate corresponded with the quantitative estimation on the presence of vitamin A in the diet (Cook, 1932). However, Ampree (1957) reported that when bixin was given to rats, it did not cause any growth on a vitamin A - free diet and there were even signs of toxic action in the organism.

Effects of annatto on mammals have been documented. Mice injected subcutaneously with annatto showed occasional sarcoma on the site of injection (Engelbreth-Holm and Iverson, 1955). Beagles showed inhibited growth and reduced food intake when 20% aqueous annatto extract was included in their diet for three months (Kay and Calandra, 1961).

MATERIALS AND METHODS

Extraction of Pigments

The extraction procedure used by Banzon and Arañez (1984) was followed. Seeds of *Bixa orellana* L. from Amadeo, Cavite were used. For the extraction of pigments, 1 L of chloroform (AR grade) for every 500 g of dried seeds was used. The seeds in chloroform were heated on a hot plate with a magnetic stirrer at a temperature of 60-61.5°C for 1 h. The chloroform (CE) extract was poured off from the seeds and evaporated to dryness using a rotary evaporator (Buchi 461) and a drying oven with a temperature of 65°C. A part of the CE was used for the genotoxicity test.

The remaining CE was treated with AR grade petroleum ether. Several changes of petroleum ether were made until the petroleum ether was already colorless. The residue after extraction with petroleum ether was dried in an oven and designated as BE. BE was subjected to TLC using Merck silica gel F 254. The flowing solvent used was glacial acetic acid and hexane (1:5, v/v). Spotting was done using 10 uL of BE.

Dominant Lethal Test

CE Residue - Petroleum Ether Extract (BE)

Sixteen male mice NMR strain, which were all sexually mature, were used as the test animals. Each of four male mice was given a dose of 400 mg BE per 1 kg body weight. Another four, were given a dose of 200 mg BE per 1 kg body weight. Each of four males was given 0.2 ml dimethylsulfoxide (DMSO) and another four, 0.2 ml distilled water. Those given DMSO and distilled water served as controls. The BE given to each male mouse was dissolved in around 0.2 ml DMSO. The BE, DMSO, and distilled water were given to the mice orally, by gavage, for 2 days. The mice were kept in plastic cages at one mouse per cage. They were given feeds containing pellets and seeds and water *ad libitum*.

Two untreated female mice were added to each cage of the treated male the day after the second treatment. Around two weeks after detection of

pregnancy by means of a vaginal plug, the female mice were sacrificed by cervical dislocation and dissected. The uteri of the dissected mice were examined for live and dead implants. The number of live and dead implants as well as the number of pregnant and non-pregnant females were recorded.

Crude Atsuete-Chloroform Extract (CE)

As a preliminary to the present study and to find out if the Dominant Lethal Test could be used to determine the genotoxicity of atsuete pigments, crude atsuete-chloroform extract (CE) was used as test material before using BE. The same procedure as above was followed. However, only 12 males were used. Mice given DMSO were the only ones included in the control. Four untreated females, instead of two, were mated to one treated male.

RESULTS AND DISCUSSION

Extraction of Pigments

CE was observed as a brick-red sticky solid while BE was a brick-red powder. BE produced only one spot on the TLC plate with an Rf value of 0.3.

Dominant Lethal Test

CE Residue - Petroleum Ether Extract (BE)

The number of pregnant females mated with males given varying concentration of BE is more or less the same (Table 1). However, more dead implants were observed in female mice mated with males given BE than that of the control indicating that BE is genotoxic. There were 2.1, 8.6, 18.3, and 20.4% dead implants in female mice mated with males given distilled water, DMSO, 200 mg BE per 1 kg body weight, and 400

Table 1. Pregnancy in female mice mated with males given varying doses of BE.

Dose(mg/1 kg body weight)	Total No. of females	Pregnant females	% Pregnancy
0 (distilled water)	8	7	87.5
0 (DMSO)	8	6	75.0
200	8	6	75.0
400	8	6	75.0

mg BE per 1 kg body, respectively (Table 2). The percentage of female mice that became pregnant and had dead implants were 14.2, 33.0, 83.3 and 100% for female mice mated with males given distilled water, DMSO, 200 mg BE per 1 kg body weight, and 400 mg BE per 1 kg body weight, respectively (Table 3).

Table 2. Dead implants in female mice mated with males given Varying Doses of BE.

Dose (mg/1 kg body weight)	Total No. of implants	No. of Live Implants	No. of Dead Implants	% Dead Implants
0 (distilled water)	47	46	1	2.1
0 (DMSO)	46	42	4	8.6
200	49	40	9	18.3
400	44	35	9	20.4

Table 3. Percent of female mice mated with males given varying doses of BE that became pregnant and had dead implants.

Dose (mg/1 kg body weight)	No. of females became pregnant	Females with dead Implants	% Dead Implants
0 (distilled water)	7	1	14.2
0 (DMSO)	6	2	33.0
200	6	5	83.3
400	6	6	100.0

Crude Atsuete - Chloroform Extract (CE)

It appeared that low dose of atsuete extract enhanced fertility in males since 75% of female mice mated with male mice given 200 mg atsuete extract per 1 kg body weight became pregnant. Only 50% of female mice mated with male mice given DMSO, which served as the control, became pregnant. The pregnancy in females mated with males given 400 mg CE per 1 kg body weight is only 25% (Table 4).

Table 4. Pregnancy in female mice mated with males given varying doses of CE.

Dose (mg/1 kg body weight)	Total No. of females	Pregnant females	% Pregnancy
0 (DMSO)	14	7	50
200	16	12	75
400	16	4	25

More dead implants were observed in female mice mated with treated males, especially those females mated with males that were given 400 mg CE per 1 kg body weight. Female mice mated with male mice given DMSO only had 5.7% dead implants; those mated with male mice given 200 mg CE per 1 kg body weight had 9.2% dead implants; while those given 400 mg CE per 1 kg body weight had 26.9% dead implants (Table 5). The results showed that DMSO was somewhat genotoxic. However,

Table 5. Dead implants in female mice mated with males given varying doses of CE.

Dose (mg/1 kg body weight)	Total No. of Implants	No. of Live Implants	No. of Dead Implants	% Dead Implants
0 (DMSO)	35	33	2	5.7
200	65	59	6	9.2
400	26	19	7	26.9

the percentage of dead implants in female mice mated with males treated with 400 mg CE per 1 kg body weight was much more than that of the control. In Fig. 1 is a dissected female mouse, with no dead implant, mated with a male given distilled water (control). In Fig. 2 is a dissected female mouse, with 3 live and 3 dead implants, mated with a male given 400 mg CE per 1 kg body weight.

Based on the study of Tanchico and West (1936) and Batoon (1978), there are two pigments in atsuete dye, bixin and orellin. Orellin is soluble in petroleum ether unlike bixin (Batoon, 1978), hence BE contains bixin while the petroleum ether extract contains orellin. The petroleum ether extract was observed as mutagenic/genotoxic by Banzon and Arañez (1984) and Arañez and Rubio (1996). According to Banzon and Arañez (1984), orellin could act as an alkylating agent since it could be converted

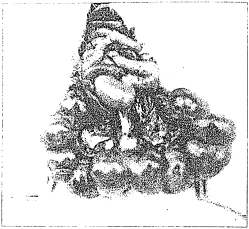


Figure 1. Dissected female mouse, with no dead implant, mated with a male given distilled water (control).

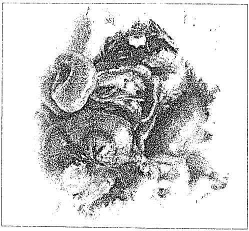
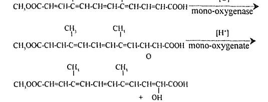


Figure 2. Dissected female mouse, with 3 live and 3 dead implants, mated with a male given 400 mg CE per 1 kg body weight.

into an epoxide by undergoing oxygen insertions at a number of double bonds. The epoxide could be protonated to produce a more reactive electrophile which could interact with DNA (Banzon and Arañez, 1984):



The above reactions could be made possible by the presence of a microsomal enzyme system in mice.

Just like orellin, bixin could probably be converted into an epoxide and acts as an alkylating agent. The functional groups in bixin and orellin are similar and both have double bonds.

Alkylated guanine may be released from DNA producing apurinic sites (Scalera and Ward, 1971). Apurinic sites may lead to strand breaks (Manson, 1980). The Dominant Lethal Test has been used to screen for agents that produce chromosomal breakage in gametic cells (Green et al., 1985). The gametic lesion that can occur in a gamete does not interfere with the ability of the gamete to fertilize or be fertilized but is lethal to the resulting embryo (Bateman and Epstein, 1971). The broken chromosomes are eventually lost, resulting in an embryo that subsequently dies in utero (Green et al., 1985).

Chromosomal aberrations, in the form of small deletions, may not be detected by the Micronucleus Test and Allium Test but could be detected by the Dominant Lethal Test. Individuals heterozygous for a deletion may not survive since the genome has been "fine-tuned" during evolution to require a specific balance or ratio of most genes (Suzuki et al., 1989). In addition to deletion, strand breaks may give rise to other kinds of chromosomal aberrations such as inversions and translocations which may not be detected by the Micronucleus Test. Chromosomal aberrations producing dicentric chromosomes could be detected by the Allium Test since they give rise to chromosome bridges. However, translocated chromosomes with only one centromere and some chromosomes with inversions may not be detected by the Micronucleus Test and Allium Test. Translocation and inversion heterozygotes may give rise to unbalanced gametes. The unbalanced gametes that may fertilize normal gametes give rise to unbalanced zygotes that tend not to survive (Suzuki et al., 1989).

Bixin could be converted to orellin and other degradation products (McKeown and Mark, 1962; Tong, 1984). To confirm that the genotoxic agents in BE were not degradation products of bixin, TLC was done on the remaining BE. Only one spot was observed as before confirming that bixin was not degraded.

SUMMARY AND CONCLUSION

The genotoxicity of the crude atsuete-chloroform extract (CE) and the CE residue - petroleum ether extract (BE) was determined by the Dominant Lethal Test.

BE was found to be genotoxic. More dead implants were observed in female mice mated with males given BE than those of the control. All female mice mated with males given 400 mg BE per 1 kg body weight had dead implants; 83.3% for those mated with males given 200 mg BE per 1 kg body weight; while only 33.0% and 14.2% of the female mice mated to males given DMSO and distilled water, respectively, had dead implants.

CE was also observed to be genotoxic. More dead implants were observed in females mated with males given CE than those of the control.

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Study on the Antifungal Efficacy of Juliflorine and a Benzene-Insoluble Alkaloidal Fraction of *Prosopis Juliflora*

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ABSTRACT

In vivo antidermatophytic activity of juliflorine and a benzene insoluble alkaloidal fraction obtained from Prosopis juliflora was tested against Trichophyton mentagrophytes infection, produced in rabbits. Topical application of juliflorine in concentration of 0.5%, 1% and 2.5% was found to heal 25%, 50% and 75% of dermatophytic lesions in three weeks respectively. Benzene insoluble alkaloidal fraction was found comparatively more effective than juliflorine.

The chemotherapeutic effects of these alkaloids were compared with Terbinafine which was found far more superior to these alkaloids against artificially produced dermatophytic infection.

KEYWORDS: Juliflorine, alkaloids, Prosopis juliflora, Trichophyton mentagrophytes, Artificial infection.

INTRODUCTION

The use of indigenous medicinal herbs for the treatment of diseases is actively practised in both Unani and Ayurvedic system of Indo-Pakistan subcontinent. Drugs derived from medicinal plants have served through the ages as the mainstay for the treatment of various diseases and human ailments. Throughout the world some 70% of the people rely on traditional herbal remedies to cure a wide variety of ailments from minor infection to asthma, dysentery, malaria etc. Scientific analysis of medicinal plants has led to the discoveries of many important drugs (Inamul-Haq 1983; Baquar 1989; Ahmad *et al.* 1992).

In view of these facts systemic investigations of medicinal plant were initiated in order to isolate the active principles of various plant materials to bring up new medically important substances, which could be used as effective therapeutic agents.

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In a large number of medicinal plants, the therapeutic value is due to the presence of alkaloids, which in certain respect ranks among the most interesting of the naturally occurring substances.

The genus *Prosopis* (mesquite) are known to possess medicinal value (Kirtikar, 1935). *Prosopis juliflora,* a shrub, grows abundantly in Sindh and Punjab provinces of Pakistan (Nasir & Ali, 1972). Juliflorine and julifloricine, the main alkaloids of *Prosopis juliflora*, have been isolated for the first time by Ahmad *et. al.* (1978) and the antibacterial and antifungal activities were reported by Khan, *et al.* (1986) and Ahmad, *et. al.* (1986; 1988a; 1989a). From *P. juliflora*, a benzene insoluble alkaloidal fraction (containing 2 major and 3 minor alkaloids) has also been isolated and reported to possess antibacterial and antifungal activities (Ahmad *et al.* 1988b; Ahmad *et al.* 1989b). Toxicity of these alkaloids have also been determined (Ahmad *et al.*1991a; 1991b; 1995).

The present work deals with the study of the therapeutic efficacy of these alkaloids against artificially produced dermatophytic infection in rabbits. Efficacy of these alkaloids were also compared with an antifungal antibiotic, the terbinafine.

MATERIALS AND METHODS

Rabbits (*Lipus capensis*-local) with body weight 1.5 - 2 Kg were used in this study. Animals were kept in animal house at 25-30°C.

Ointments of the alkaloids in the following concentrations were prepared in petroleum jelly:

1. Juliflorine 0.1%, 0.5%, 1% and 2.5%.
2. Benzene insoluble alkaloidal fraction 0.1%, 0.5%, and 1%.
3. Terbinafine 0.1% and 0.5%.

The above preparations were applied on the infected skin of the rabbits.

Infective material

In order to study the effectiveness of the alkaloids, *in vivo* dermal infection using *Trichophyton mentagrophytes* (clinical isolates) were produced in rabbits. The inocula of the culture was prepared as follows:

To prepare the fungal inoculum, plates of Taplin agar (Merck) were inoculated and incubated at 32°C for 7 days. The growth was then scraped off with a spatula under sterile condition, and finely dispersed in Sabouraud dextrose broth (Oxoid) by means of glass homogenizer. Inocula of *T. mentagrophytes* was adjusted by turbidimetry (final transmission read on absorption was 65%). The inoculum was tested for possible contamination by inoculating in Sabouraud dextrose agar plates.

Artificial Infection

The shoulder, hips and back of rabbits were first shaved with razor and the area was disinfected with alcohol. The 0.1 ml fungal inoculum was rubbed on the skin in an area of 9cm^2 with glass rod. The infected rabbits were kept at room temperature (25-30°C) for 3-7 days. The infection was confirmed by microscopy and culture.

Therapeutic trials

The alkaloidal formulation was applied locally with spatula on the infected area of the skin of two rabbits with fungal infection. Two rabbits were also kept as control and compared with those under treatment.

Mycological Evaluation

This was done by taking skin scraping for microscopy and culture and; also by performing the hair follicle test.

The skin scrapings were taken by sterile scalpel after disinfection with alcohol; and were examined microscopically by using 10% KOH. Some material was also inoculated on Mycobiotic agar (Difco) and plates were incubated at 32°C for 1-2 weeks and examined for fungal growth.

Hair follicle test

This test provides a measure of the incidence of residual infection in the skin foci of individual laboratory animal. After three weeks of treatment, all the animals were examined. About 10 hair per animal were removed with sterile forceps, 5 hair were examined microscopically by using 10% KOH and remaining 5 hair were inoculated on Mycobiotic agar (Difco). These plates were incubated at 32°C for 1-2 weeks and examined for fungal growth.

The effectiveness of alkaloids in reducing the number of positive sample per treated group is expressed as percentage of that in the untreated control group. viz:

Effectiveness = 100 - (T x 100)/K %

Where T = Average number of positive samples in the particular group tested.

K = Average number of positive samples in the control group.

Clinical Evaluation

The degree of local change in each animal was noted on the 3rd and 11th days after infection. The effectiveness of the alkaloids were assessed on the 11th, 18th and 25th day after infection and expressed as percentage of the degree of infection in the untreated group.

RESULTS AND DISCUSSION

The juliflorine and benzene insoluble alkaloidal fraction were studied for their therapeutic efficacy in artificially produced superficial skin infection in rabbits. Infection was produced by rubbing freshly isolated culture of *T. mentagrophytes* on to 9cm^2 shaved skin. Treatment was started after third day of infection with 0.1%, 0.5% and 2.5% of the alkaloids; and 0.1 and 0.5% Terbinafine prepared in petroleum gel. Each concentration was applied locally onto the infected sites of the rabbits. Clinical efficacy of the alkaloids was determined by healing of lesions; and microbiological efficacy was determined by culture and microscopic examination of the skin scraping and hair filaments from infected areas. Results of therapeutic efficacy are shown in Table-1.

Topical application of juliflorine in concentration of 0.5%, 1% and 2.5% was found to heal 25%, 50% and 75% of dermatophytic lesions in three weeks respectively. The microbiological efficicacy of juliflorine in concentrations of 0.1%, 0.5%, 1% and 2.5% to dermatophytic infection was found 0%, 20%, 60% and 80% respectively (Figure-1). Recurrence was observed at 1% concentration but not at higher concentration (Table-1). Benzene insoluble alkaloidal fraction was found comparatively more effective than juliflorine. Healing was slightly faster than juliflorine and also recurrence of infection was nil at 1% and 2.5% concentration. Juliflorine and benzene insoluble alkaloidal fractions were found effective at 2.5% concentration, but these concentrations were also found toxic. The standard antibiotic terbinafine was found much more superior to these alkaloids in artificially produced skin infections (Figure-2).

The study shows that juliflorine and benzene insoluble alkaloidal fraction are effective in dermatophytic infections and generally dose-related clinical and microbiological efficacies are noted. Juliflorine in concentrations 0.5-2.5% were found effective in curing 25-75% of dermatophytic lesions in three weeks. Microbiological efficacy of juliflorine was also noted in dermatophytic infections (Figure-1).

Clinical examination shows that at high concentration the characteristic lesions heals or subsides. But continuous application of these alkaloids makes the skin sensitive which resulted in inflammation, induration etc. and the risk of secondary infection increases with resistant strains (Table-1).

All these alkaloids have also been screened for mutagenicity by Ames test. They were found non-mutagenic up to 500 μg/plate, hence these are non-carcinogenic (Khan, *et al.* 1986; Ahmad *et al.* 1991a; Ahmad *et al.* 1995). Although these alkaloids have shown some encouraging results, they need to undergo carefully controlled trials in order to evaluate their usefulness in daily medical practice.

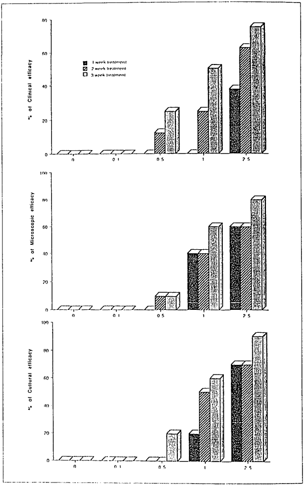
Table 1. The chemotherapeutic efficacy after topical application of ointment of Juliflorine, a benzene insoluble alkaloidal fraction of *Prosopis juliflora* and Terbinafine in dermatosis model *Trichophyton mentagrophytes* in rabbits.

		EFFECTIVENESS										
Compounds		Clinical % cured			Mycological % cured							
		1 Week	2 Weeks	3 Weeks	1 Week		2 Weeks		3 Weeks		Reinfection	Remarks
					M	C	M	C	M	C		
Control		0	0	0	0	0	0	0	0	0		
Juliflorine	0.1 %	0	0	0	0	0	0	0	0	0		
	0.5 %	0	12.5	25	0	0	10	0	10	20	+	
	1.0 %	0	25	50	40	20	40	50	60	60	+	a
	2.5 %	37.5	62.5	75	60	70	60	70	80	90	-	b
Benzene insoluble alkaloidal fraction	0.1 %	0	0	0	10	0	10	0	10	20	+	0
	0.5 %	25	25	37.5	30	20	40	30	60	50	+	a
	1.0 %	25	50	87.5	30	40	40	60	80	70	-	b
	2.5 %	25	87.5	100	50	40	70	70	90	100	-	c
Terbinafine	0.1 %	100	**	**	90	100	100	100	**	**		
	0.5 %	100	**	**	100	100	**	**	**	**		

* Mycologically cured % or Effectiveness = $100 - \frac{T^{*}}{K} 100$ %

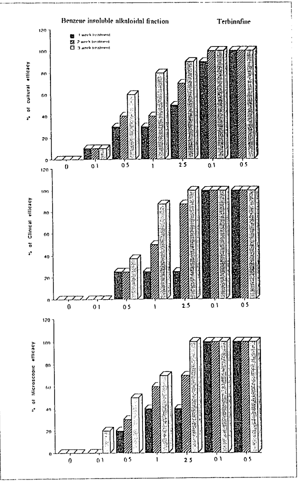
T = Average no. of positive sample in the Test study
K = Average no. of positive sample in the Control study

M = Microscopy
C = Culture
** = Treatment discontinued
a = Slight hardening of skin
b = Slight toxic effect was seen. Skin became slight inflamed
c = T. mentagrophytes inhibited but skin became susceptible and secondary infection was observed.
N.B. In several hair, disintegration of fungal filaments was also seen.



Percentage of Juliflorine in Petroleum jelly.

Figure 1. Therapeutic efficacy of juliflorine against dermatophytic infection.



% of benzene insoluble alkaloidal fraction and Terbinafine in Petroleum jelly.

Figure 2. Therapeutic efficacy of benzene insoluble alkaloidal fraction and Terbinafine against dermatophytic infection.

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An Antimutagenic Monoterpene from *Malachra Fasciata* (Malvaceae)

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KEYWORDS
antimutagen, loliolide, *Malachra fasciata*, Malvaceae, monoterpene, stigmasterol

ABSTRACT

A monoterpene was isolated from the leaves of Malachra fasciata by gravity column chromatography. Its structure was elucidated by extensive 1D and 2D NMR spectroscopy. It was identified as loliolide by comparison of its ^1H and ^{13}C NMR spectral data with those found in the literature.

The compound was tested for its antimutagenicity potential by the use of the Micronucleus test. Results of the study indicated a 64.4% reduction in micronucleated polychromatic erythrocytes induced by mitomycin C, when loliolide at a dosage of 14.8 mg/kg was administered to mice of the Swiss strain. Another isolate from the leaves of the plant was stigmasterol which structure was determined by comparison of its ^1H NMR spectral data with those found in the literature.

INTRODUCTION

Malachra fasciata or situyo is a shrub found throughout the Philippines at low altitudes. Poultices of the leaves are applied to ulcers and other sores. The roots and leaves may serve as emolients and may be considered specific against haemorrhoids, fevers and impotency and also as a general tonic. A decoction of the leaves is used for treatment of gonorrhoea and rheumatism and as a demulcent and diuretic. The plant was also reported to have antitumor properties (Quisumbing, 1951).

There is no reported chemical study on Malachra fasciata. This is the first report on the isolation, structure elucidation and antimutagenicity studies on loliolide (1) from the leaves of M. fasciata. Loliolide has been identified as a constituent of Lolium perenne (Manske, 1938), Digitalis pupurea (Satoh and Wada, 1956), and Fumaria officinalis)Satoh and Wada, 1964). We also report the isolation of stigmasterol (2) from the leaves of the plant. A previous study showed that stigmasterol at a dosage of 170 mg/kg reduced the number of micronucleated polychromatic erythrocytes induced by Mitomycin C by 79% (Ragasa, et. al., 1995).

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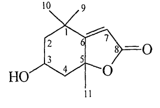

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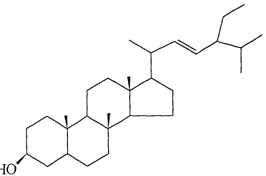

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RESULTS AND DISCUSSION

The chloroform extract of the air-dried leaves of Malachra fasciata afforded loliolide (1) and stigmasterol (2). The structure of loliolide was elucidated by NMR (^1H, ^{13}C, COSY, HMQC and HMBC) spectroscopy and the ^{13}C NMR spectral data of 1 was comparable to those of loliolide found in the literature [Ravi, et. al., 1982]. The structure of 2 was determined by comparison of its ^1H NMR spectral data with those of an authentic sample of stigmasterol [Zulueta, 1994].

The ^1H NMR spectrum of 1 indicated resonances for olefinic hydrogen on a conjugated double bond at δ 5.70 (1H, s), a carbinyl hydrogen of an

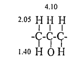
alcohol at δ 4.10 (1H, m) and three methyl singlets at δ 1.26 (3H, s), δ 1.32 (3H, s) and δ 1.66 (3H, s). The ^{1}H NMR spectral data of **1** is presented in Table 1.

Table 1. 300 MHz ^{1}H, ^{13}C and HMQC spectral data of 1 in $CDCl_3$.

Carbon	^{13}C shift, d	1H shift, d
C-1	35	-
C-2	50	1.40, 2.05
C-3	65	4.10
C-4	48	1.55, 2.55
C-5	86	-
C-6	181	-
C-7	114	5.70
C-8	172	-
C-9	30	1.37
C-10	25	1.32
C-11	26	1.67

The ^{13}C NMR spectrum gave evidence to the presence of eleven carbon atoms in **1**. The resonance at δ 172 was attributed to the carbonyl carbon of a lactone, while those at δ 181 and 114 indicated one carbon-carbon double bond. Probably the quaternary carbon at δ 181 is further deshielded due to the steric effect of neighboring methyl groups. Two carbons singly bonded to oxygen were deduced from the resonances at δ 87 and 65, while three methyl groups were assigned to the resonances at δ 30, 26 and 25. The ^{13}C NMR spectral data of **1** is summarized in Table 1.

The COSY spectrum of **1** indicated only one isolated spin system. The hydrogen at δ 2.05 is coupled to the protons at δ 1.40 and 4.10. The latter proton is further coupled to the hydrogens at δ 2.55 and 1.55. Thus, the following is an isolated spin system deduced from COSY.



The HMQC spectrum indicates the hydrogens that are directly bonded to carbons as follows. The olefinic hydrogen at δ 5.70 is bonded to the carbon at δ 114, while the carbinyl hydrogen at δ 4.10 is attached to the carbon at δ 65. The methylene protons at δ 2.05 and 1.40 are bonded to the carbon at δ 50, while those at δ 2.55 and 1.55 are attached to the carbon at δ 48. The methyl protons at δ 1.58, 1.37 and 1.32 are bonded

to the carbons at δ 26, 30 and 25, respectively. These data are summarized in Table 1.

The structure of 1 was deduced from the HMBC spectrum summarized in Table 2. This spectrum indicates long-range correlations between carbons and protons two to three bonds away. The carbon at δ 181 is long-range correlated to the hydrogens at δ 5.70, 2.55, 1.55, 1.67, 1.32, 1.37, 2.04 and 1.40. Long-range correlations were observed between the carbon at δ 172 and the olefinic proton at δ 5.70. Further correlations were deduced from the carbon at 86 and the hydrogens at δ 2.55, 1.67 and 1.55. The carbon at δ 64 is long-range correlated to the protons at δ 2.55, 2.05, 1.67 and 1.40. Additional correlations were attributed to the carbon at δ 50 and the hydrogens at δ 2.55, 1.55, 1.37 and 1.32. The carbon at δ 47 is long-range correlated to the protons at δ 2.05 and 1.40, while the one at δ 35 is correlated to the hydrogens at δ 1.37, 1.32, 1.40, 2.05 and 5.70. The methyl carbon at δ 30 is long-range correlated to the protons at δ 1.32 and 1.40, while another methyl carbon at δ 25 is correlated to the hydrogens at δ 1.37 and 1.40. The third methyl carbon at δ 26 is long-range correlated to the hydrogen at δ 1.55. All long-range correlations observed were consistent with the structure of 1.

Table 2. 300 MHz ^{13}C-^{1}H long-range (HMBC) spectral data of 1.

Carbon	Hydrogens
C-1	H-3a, H-2b, H-7, H-9, H-10
C-2	H-4a, H-4b, H-9, H-10
C-3	H-2a, H-2b, H-4a, H-4b
C-4	H-2a, H-2b
C-5	H-4a, H-4b, H-7, H-11
C-6	H-2a, H-2b, H-4a, H-4b, H-7, H-9, H-10, H-11
C-7	
C-8	H-7
C-9	H-2a, H-10
C-10	H-2a, H-9
C-11	H-4

Literature search revealed that 1 is loliolide. Confirmatory evidence was the ^{13}C NMR spectral data of 1 and loliolide (Ravi, et. al., 1982). The data matched in all essential respects.

The plant was reported to have antitumor property. Because of a strong correlation between antitumor and antimutagenic activities, the antimutagenicity potential of 1 was determined by the Micronucleus test. Results of the study presented in Table 3 indicated that at a dosage of 14.8 mg/kg, 1 reduced the number of micronucleated polychromatic erythrocytes (MPCE) induced by mitomycin C by 64.4%. Statistical

analysis using the T-test indicated a significant reduction of MPCE at $\alpha = 0.01$. Thus, 1 is an antimutagen.

Table 3. Effect of 1 on the number of micronucleated polychromatic erythrocytes induced by mitomycin C.

Sample	Ave. MPCE/1000 PCE	% Reduction in MPCE
exp'l mouse 1	2.3	65.2
exp'l mouse 2	2.3	65.2
exp'l mouse 3	2.0	69.7
exp'l mouse 4	2.3	65.2
exp'l mouse 5	2.7	59.1
Average	2.3 ± 0.13*	64.9
Control mouse 1	6.0	
Control mouse 2	6.7	
Control mouse 3	7.0	
Control mouse 4	6.7	
Control mouse 5	6.7	
Average	6.6 ± 0.20*	

*Average of 15 slides

The structure of 2 was deduced by comparison of its ^1H NMR spectral data with those of an authentic sample of stigmasterol (Table 4) (Zulueta, 1994). The spectra matched in all essential respects, and hence, confirming that 2 is stigmasterol. Antimutagenicity test was not conducted on 2 since a previous study already reported its antimutagenic activity (Ragasa, et. al., 1995).

Table 4. A comparison of 1H NMR spectral data of 2 and stigmasterol from an authentic sample (Zulueta, 1994).

Protons	^1H Shift of 2, d	^1H Shift, d from an Authentic Sample [Zulueta, 1994]
H-3	3.51	3.52
H-6	5.08	5.09
H-18	0.69 (3H)	0.70
H-19	1.00 (3H)	1.00
H-21	1.02 (3H)	1.02
H-22, H-23	5.33	5.33
H-26, H-27	0.85 (6H)	0.85
H-29	0.82 (3H)	0.82

EXPERIMENTAL

NMR spectra were recorded in $CHCl_3$ on a 300 MHz Bruker AMX spectrometer. Silica gel 60 (70-230 mesh) was used for column chromatography and plastic backed plates coated with Si gel F_{254} were used for TLC. Plates were visualized by spraying with vanillin:H_2SO_4 and warming. The number of MPCE was counted with the use of a Zeiss microscope.

The plant sample was collected from Sta. Barbara, Pangasinan, Philippines in August 1995. It was identified by the botanists at the National Museum as Malachra fasciata.

Air-dried leaves of the plant samples (500 g) were soaked in 2 L of $CHCl_3$ for 3 days, then filtered. The filtrate was concentrated under vacuum to afford a crude extract (20 g) which was treated with 4% aqueous $Pb(OAc)_2$ to precipitate the pigments (Padolina, et. al., 1974). The treated extract (1 g) was subjected to gravity column chromatography (dry packing). The solvent system was based on step gradient technique, starting with $CHCl_3$, then Me_2CO in $CHCl_3$ (10% increments). The 30% Me_2CO in $CHCl_3$ fraction was rechromatographed in 20% Me_2CO in $CHCl_3$ to afford 1 (colorless oil, 3.85 mg). The $CHCl_3$ fraction was rechromatographed in $CHCl_3$ to afford 2 (20 mg, colorless crystals, m. pt. 170 °C).

Antimutagenicity Test

The test compounds (14.8 mg/kg mouse) dissolved in DMSO (7.5 mL/kg mouse, solvent control) were administered simultaneously with mitomycin C (2.75 mg/kg mouse, positive control) to mice of the Swiss strain (source: DOST). For the control, only mitomycin C and DMSO were administered orally to the mice. Five mice replicates were tested for each compound and control. The second administration was carried out after 24 h. The mice were killed by dislocation of the neck, 6 h after the second administration. Blood from the bone marrow was smeared on slides (three per mouse). The slides were stained with May Grunwald and Giemsa solutions [Schmid, 1972]. The numbers of MPCE/1000 PCE were counted by the use of a high power Zeiss microscope.

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Two New Additions to the Udoteaceae (Chlorophyta, Bryopsidales) of the Philippines

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ABSTRACT

The genus Rhipilia Kützing (Chlorophyta, Udoteaceae), represented by R. orientalis A. Gepp & E.S. Gepp, is reported for the first time from Philippine marine waters. Another species of the Udoteaceae, Udotea polychotomis Cordero, is also reported herein as a new addition to the Philippine marine algal flora. Vegetative morphology of these two entities is described and compared with other related taxa. A synoptic key to the Philippine genera of the Udoteaceae is also presented.

INTRODUCTION

Species of the Udoteaceae are a group of marine green algae exhibiting a pantropical distribution. Many members are calcified and are thus considered important contributors to reef formation. They are also well represented in the fossil record dating as far back as the Lower Devonian or about 390 million years ago (Hubmann 1994).

In Philippine marine waters, the Udoteaceae is represented by seven extant genera (Silva et al. 1987, Littler & Littler 1990), namely: Avrainvillea *Decaisne,* Boodleopsis *A. Gepp & E.S. Gepp,* Chlorodesmis *Harvey & Bailey,* Rhipidosiphon *Montagne,* Rhipiliopsis *A. Gepp & E.S. Gepp,* Tydemania *Weber-van Bosse and* Udotea *Lamouroux. An unidentified member of an eighth genus,* Cladocephalus *Howe was reported by Taylor (1966) but owing to the fragmentary nature of the single specimen examined, its identity cannot be ascertained, hence this record remains, at best, doubtful. Originally described as a common element of the Caribbean marine flora, the genus* Cladocephalus *has only been reported from the western Indian Ocean (Silva et al. 1996) but nowhere from the Pacific.*

This paper reports the occurrence of yet another genus of the Udoteaceae, and a species of the genus Udotea recorded for the first time from Philippine waters.

MATERIALS AND METHODS

The specimens examined in this study were collected by the Smithsonian Institution Philippine Expedition (SIPHILEX) from various sites in the Cuyo Islands, west central Philippines between May 21-26, 1978. More

detailed descriptions of the collecting stations are given in Liao (1987). Replicate specimens have been deposited in the following herbaria: BM, GUAM, L, MICH, NY, P, PNH, UC, US and the herbarium of the Marine Laboratory, Silliman University, Dumaguete, Philippines [standard herbarium abbreviations follow Holmgren et al. (1990)].

Fragments from calcified specimens were removed and observed under the dissecting microscope while being decalcified in 10% HCl. Anastomosing filaments were carefully teased apart using fine point needles or forceps and mounted on clean glass slides. Sections were stained with aqueous aniline blue solution. Measurements were made using precalibrated ocular micrometers. Illustrations were made with the use of a Wild camera lucida attachment.

RESULTS AND DISCUSSION

The genus *Rhipilia* Kützing, represented by the species *Rhipilia orientalis* A. Gepp & E.S. Gepp, is reported for the first time from the Philippines. It is widely distributed in the warm waters of the Indo-Pacific (Taylor 1950, Coppejans & Prud'homme van Reine 1989, Silva et al. 1996). Its occurrence in the Philippines is expected as the Philippine marine flora is oftentimes characterized as one of the centers of diversity of the rich Indo-Pacific marine biota (Lüning 1990). The small, inconspicuous nature of the thalli belonging to the genus *Rhipilia* might account for their being easily overlooked specially by non-phycologists.

Rhipilia orientalis **A. Gepp & E.S. Gepp** Figs. 1 and 2

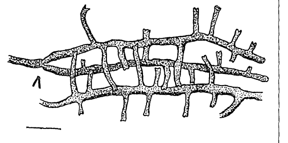

Figure 1. *Rhipilia orientalis*. Section from thallus slightly teased apart to show adherent filaments producing mostly perpendicularly issued determinate lateral branches. Notice the evenly placed supradichotomal constrictions (arrowhead). Scale bar = 150 mm

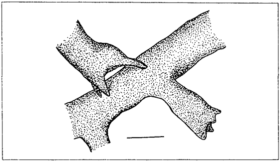

Figure 2. ***Rhipilia orientalis.*** **Three-pronged tenaculum attached to a neighboring filament (left) and a four-pronged free tenaculum (right). Scale bar = 50 µm**

A. Gepp & E.S. Gepp 1911: 57, 140, pl. 16, figs. 134a and b, 135, 136; Taylor 1950: 72; Coppejans & Prud'homme van Reine 1989: 131, pl. 6, figs. 1-18.

Plants funnel-shaped, brownish green, stipitate, small, up to 2.5 cm tall; stipe 1 cm long, made up of compact filaments, gradually expanding distally into a fan-shaped to infundibuliform frond, to 2 cm wide, with slightly fimbriate to entire margins, consisting of loosely interwoven, dichotomously branched filaments, to 60 µm diameter, commonly bearing equally levelled constrictions above a dichotomy; main filaments rarely sinuose at some points, bearing numerous determinate lateral branches of variable length, usually borne perpendicularly on both sides, with 2-4 pronged tenacula at the tips which are either free or attached to other filaments.

Type locality: Pulau Sebangkatan, Borneo

Specimens examined: 78 EM-11 (158-162, 263, 271, 277, 280, 285), channel marker near the entrance to the Cuyo municipal wharf, Cuyo Island, Palawan province, 10°51'24"N, 120°59'36"E, 0.6-3.0 m deep, sandy bottom with scattered rocks and large coral heads, water column with some suspended particles causing slight turbidity, 21 May 1978.

Remarks: Members of the genus *Rhipilia* are generally inconspicuous, growing on dead coral branches, which is probably why they are often overlooked by non-specialists. They occur singly most of the time (e.g., *Rhipilia orientalis*) but could also produce multiple erect axes (e.g., *Rhipilia penicilloides* N'Yeurt & Keats 1997).

Doty (1954) first observed that there seems to be a mutual distributional range exclusion phenomenon seen between *Rhipilia* and the pantropical brown algal genus *Sargassum* C. Agardh (Fucales, Sargassaceae). He observed during a survey of various Pacific islands that species of *Sargassum* were recorded only around high islands made of igneous substrata where no species of *Rhipilia* were found. Interestingly, the same phenomenon has been recorded in the Marshall Islands (Dawson 1956, 1957).

In the present study, *Rhipilia* was found only in one collecting site, the entrance to the Cuyo municipal wharf, where no *Sargassum* was collected. The latter was not encountered in other sampling sites on the same island, but only in the outlying islands and in sparse quantities. Tsuda & Tobias (1977) recorded *Rhipilia* and *Sargassum* on opposite sides (SE and W, respectively) of Anatahan Island in the northern Marianas. Samples were, however, collected on different years.

While it is plausible that substrata differences between high and low islands may account for the range exclusiveness of the two genera as first proposed by Doty (1954), other determining factors may be responsible. If *Rhipilia* and *Sargassum* are to occur together in the same habitat while discharging spores of different morphologies and settling abilities, their chances of survival would be unequal even if the available substrate type and other prevailing environmental conditions are relatively uniform. The mutual exclusion process would probably be more satisfactorily explained, though speculatively, by restrictive chemical factors such as allelopathy. Many temperate brown algae produce polyphenolic secondary metabolites (Steinberg & van Altena 1992) but less so in tropical fucoids such as *Sargassum* (Van Alstyne & Paul 1990). Phenolic substances are known to inhibit fouling by epiphytes (Al-ogily & Knight-Jones 1977) and may be released in minute amounts to the water column. However, Harlin (1987) pointed out that the metabolic cost of synthesizing an allelochemical and the dilution phenomenon in the aquatic medium do not seem to present much evolutionary significance to the releasor. Although well documented among terrestrial plants, allelopathy remains poorly studied among marine plants. The interesting hypothesis of Doty (1954) may remain so until more evidence is brought to the fore.

Another member of the Udoteaceae reported for the first time from the Philippines is *Udotea polychotomis* Cordero. This species was first described from the Caroline Islands and is reported for the first time outside its type locality. The present report extends its distributional range further to the west. This alga may be more widespread than previously thought.

Udotea polychotomis **Cordero**

Cordero 1974: 243, figs. 1-14.

Thalli moderately calcified, to 6 cm tall; rhizoidal holdfast small, to 5 μm diameter, commonly coated with sand particles; stipe 0.5-1.0 cm long (rarely to 2.5 cm), to 1.5 mm broad, terete, heavily calcified, polysiphonous, composed of filaments 40-45 μm diameter, densely pigmented, issuing numerous lateral appendages, bilaterally arranged, perpendicular to main filament, almost always equidistant from one another, may bear a distinct constriction near its base, becoming densely digitate towards the distal blunt tips, forming a compact cortical layer; frond to 4.5 cm across (commonly between 2-4 cm), flat, generally simple, rarely bilaminate, faintly zonate, sometimes with shallow longitudinal folds on the surface, margins entire, slightly crenulate, oftentimes with numerous proliferations, basal margin cuneate to auriculate; apical filaments 35-50 μm diameter, loosely arranged, branching commonly dichotomous, sometimes regularly or unevenly trichotomous, bearing uneven constrictions above the point of branching and elsewhere in the filaments, without lateral appendages, tips rounded; mid-frond filaments branching dichotomously, bearing few lateral appendages, short, branching once or twice at most.

Type locality: Caroline Islands

Specimens examined: 78 EM-12 (82-91, 94), NE side of Bisucay Island, Palawan province, 10°49'42"N, 120, 59'00"E, 0.3-3.0 m deep, sandy bottom with seagrass community and scattered dead staghorn corals, water column moderately turbid, 22 May 1978; 78 EM-14 (2-7), S end of Bisucay Island, Palawan province, 10°48'36"N, 120°58'24"E, 0.5-5.0 m deep, volcanic rocks forming surge channels among seagrass community, 22 May 1978; 78 EM-16 (91), SW end of Canipo Island, Palawan province, 10°58'39"N, 120°56'30"E, 0.5-5.0 m deep, sandy bottom with coral rubble, 23 May 1978; 78 EM-19 (75-85), E side of Tagauayan Island, Palawan province, 10°58'15"N, 121°13'42"E, 0.3-2.0 m deep, seagrass community with scattered coral rubble and sandy bottom, 25 May 1978; 78 EM-43 (37), W side of Putik Island, Palawan province, 3.0-4.6 m deep, 22 May 1978.

Remarks: The present collections resemble *Udotea orientalis* A. Gepp & E.S. Gepp, *U. indica* A. Gepp & E.S. Gepp, and *U. palmetta* Decaisne in external morphology. In fact, the different diagnostic features of the present materials answer the description of *U. orientalis* except for some trichotomously branching apical filaments encountered in them. It is on the sole basis of trichotomy that the specimens under study are classified under *U. polychotomis* by the researcher.

In describing *U. polychotomis*, Cordero (1974) indicated that the trichotomous nature of this species may merit recognition at the generic level. However, *U. glaucescens* Harvey, described from the Friendly Islands (present-day Tonga Islands), was observed to also have filaments that are "dichotomously or rarely trichotomously branched," among other

features (Gepp & Gepp 1911: 113). Tseng & Dong (1975) in their study of *Udotea* species from the Xisha Islands (Paracel Islands) in the South China Sea described six new species, three of which possess trichotomously branched filaments, namely: *U. fragilifolia* Tseng & Dong, *U. tenax* Tseng & Dong, and *U. xishaensis* Tseng & Dong. A careful study is necessary using type materials of these taxa to ascertain the integrity of the trichotomous nature of filaments among species of *Udotea*. An emended description of *U. orientalis* may be in order if its type specimen is found to contain trichotomously branched filaments.

This first report of *Rhipilia* from Philippine waters brings the total number of local genera within the Udoteaceae to eight. A key to the eight genera as they occur in the Philippines is appended below.

1. Thalli with calcified portions ------------------------------ 2
1. Thalli lacking calcified portions --------------------------- 3
    2. Blades unistratose, siphons smooth, lacking corticating projections, stipe consisting of a single filament ---------------- Rhipidosiphon
    2. Blades multistratose, differentiated into cortical and interior layers, siphons usually beset with corticating projections, stipe consisting of a core of filament bundles ------------------ Udotea
3. Thalli forming undifferentiated green cushions ------------ *Boodleopsis*
3. Thalli differentiated into upright blades or tufts and rhizoidal cushions ----- 4
    4. Upright portions consisting of free filaments or glomeruli -------- 5
    4. Upright portions consisting of filaments that are anastomosing or adherent at certain points ------------------------ 6
5. Filaments free, issued randomly from basal cushion ------- *Chlorodesmis*
5. Filaments laxly anastomosing, issued from erect primary axis in verticillate manner forming glomeruli -------------------------------- *Tydemania*
    6. Blades commonly flabellate, capituliform at times, internal filaments anastomosing ------------------ *Avrainvillea*
    6. Blades peltate to infundibuliform, internal filaments adherent at certain points -------------------------- 7
7. Adjacent filaments attaching by means of tenacula or hapters at the tips of branches -- *Rhipilia*
7. Adjacent filaments adhering by means of cohesion papillae arising directly from filament wall ---------------------------------- *Rhipiliopsis*

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Liter	L
Milliliter	ml
Cubic meter	m^3
Energy and Work	KJ
Kilojoule (replace calorie in dietetics)	
<u>Mass</u>	
Kilogram	kg
Gram	g
Ton (metric ton)	t
Milligram	mg
<u>Time</u> (same units used in both Metric and English System)	
Day	d
Hour	h
Minute	min
Second	s
<u>Amount of substance</u>	
Mode	mole
<u>Temperature</u>	
Degree celsius	°C